STUDIES ON INTERACTION OF *Rhizobium leguminosarum* bv. *trifolii* AND *Trichoderma harzianum* ON NITROGEN FIXATION IN SHAFTAL CROP (*Trifolium resupinatum* L.)

Dissertation

Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in
MICROBIOLOGY
(Minor Subject : Biochemistry)

By

Pooja Khanna
(L-2001-BS-50-D)

Department of Microbiology
College of Basic Sciences and Humanities
PUNJAB AGRICULTURAL UNIVERSITY
LUDHIANA-141 004
2005

Dedicated To

My

Affectionate Parents

Who taught me to dream and gave me roots of responsibility and wings of independence

CERTIFICATE-I

This is to certify that the dissertation entitled, **"Studies on interaction of *Rhizobium leguminosarum* bv. *trifolii* and *Trichoderma harzianum* on nitrogen fixation in Shaftal crop (*Trifolium resupinatum* L.)"** submitted for the degree of **Doctor of Philosophy,** in the subject of **Microbiology** (Minor subject : **Biochemistry**) of Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Pooja Khanna (L-2001-BS-50-D)** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

Dhahal 25/4/05

Major Advisor
(Dr. V.P.S. Chahal)
Professor of Microbiology
Deptt. of Microbiology
Punjab Agricultural University
Ludhiana - 141004

CERTIFICATE-II

This is to certify that the dissertation entitled, **"Studies on interaction of *Rhizobium leguminosarum* bv. *trifolii* and *Trichoderma harzianum* on nitrogen fixation in Shaftal crop (*Trifolium resupinatum* L.)"** submitted by **Pooja Khanna (L-2001-BS-50-D)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in the subject of **Microbiology** (Minor subject : **Biochemistry**) has been approved by the Student's Advisory Committee after an oral examination of the same, in collaboration with an External Examiner.

21/4/05

Major Advisor
(Dr. V.P.S. Chahal)

21/04/05

External Examiner

Dr.P.K.Sharma
Associate Professor
Deptt.of Microbiology, COBS
CCS, Haryana Agril. University
Hissar.

21/04/05

Head of the Department
(Dr. P.K. Khanna)

Head
Microbiology
LUDHIANA.

10/5

Dean, Post graduate studies
(Dr. Darshan Singh)

ACKNOWLEDGEMENTS

Foremostly, it is prerogative to express heartfelt thanks to 'ALMIGHTY LORD", the merciful and compassionate with whose benign blessings, grace and glory I have been able to fulfill this endeavour.

With profound reverence, I deem it to be a rare privilege to record my deep sense of gratitude to my worthiest ever teacher-guide Dr. V.P.S. Chahal, Professor of Microbiology, Punjab Agricultural University, Ludhiana for his judicious and dextrous guidance, incessant encouragement, deep scientific vision, affectionate gratitude and constant supervision throughout the period of my course and research work and giving final shape to this manuscript.

I acknowledge and express my sincere thanks to my advisory committee, Dr. Joginder Singh, Senior Biochemist, Department of Biochemistry and Chemistry, Dr. R.S. Kahlon (Retd.), Professor of Microbiology, Department of Microbiology, Dr. S.M. Beri (Senior Forage Breeder), Department of Plant Breeding, Dr. (Mrs.) P.P.K. Chahal, Senior Plant Pathologist, Department of Plant Pathology, Dr. (Mrs.) Maninder K. Arora, Microbiologist, Department of Processing Food Engineering, Dr. G.S. Dhillon, Professor of Microbiology, Department of Microbiology for the planning of my course and research work and providing constructive inspiration for writing this manuscript. I owe my sincere thanks to Dr. P.K. Khanna, Senior Mycologist and Head of Microbiology and other members of the Department for providing to me necessary research facilities.

My special thanks to Dr. R.S. Uppal, Associate Professor, Department of Agronomy, PAU, Ludhiana who provided me timely field facilities.

I owe my sincere thanks to Mr. R.S. Khanna, Department of Soils who provided me help during course of experimentation.

I cherish with appreciation the jovial and vivacious company of my friends Monika, Kamaljeet, Priya, Manraj, Shivika and Mandeep. I feel deeply beholden when I recall the whole hearted warmth, affection and substantial help that came from my friends. Their company proved an asset to me at all stages. I pay my profound regards to Mrs. Ashu Chopra and Mrs. Lashmi who accommodate me when I reached them for help. Laboratory Staff especially Mr. Gulzari Lal deserves my thanks for his help. I owe my thanks to Mr. Deepak and Mr. Beer Bahadur (Ekta Computer Centre) for meticulous setting of this manuscript.

No available words could be traced in the presently available lexicon to acknowledge blessings, devotion, selfless sacrifices, love, warmth, affection and unflinching support extended by my parents whose ebullient inspiration have bought me up to this stage of my career and enabled me to persue my studies with unfailing zeal. It gives me immense pleasure to acknowledge all unrelenting support, enormous love and affection from my brothers Sumit and Puneet and Bhabhi Mansi. Their company cheered me in all moments and made all things smoother.

All may not be mentioned but none is forgotten.

Pooja Khanna

(POOJA KHANNA)

Title of the dissertation	:	Studies on interaction of *Rhizobium leguminosarum* bv. *trifolii* and *Trichoderma harzianum* on nitrogen fixation in Shaftal crop (*Trifolium resupinatum* L.)
Name of the Student	:	Pooja Khanna
and Admission No.	:	(L-2001-BS-50-D)
Name and Designation of Major Advisor	:	Dr. V.P.S. Chahal Professor of Microbiology
Major Subject	:	Microbiology
Minor Subject	:	Biochemistry
Degree to be Awarded	:	Ph.D.
Year of award of degree	:	2005
Total pages in dissertation	:	125
Name of University	:	Punjab Agricultural University Ludhiana

ABSTRACT

Ten isolates of *Rhizobium leguminosarum* bv. *trifolii* and four isolates of *Trichoderma harzianum* were obtained from nodules and rhizospheric soil of Shaftal (*Trifolium resupinatum* L.) respectively. *Rhizobium* isolates were screened in pots using sterilized soil for their efficacy to fix nitrogen on the basis of number of nodules, their dry weight, leghaemoglobin content of nodules, fresh and dry weights of shoot and root, chlorophyll content of leaves, nitrogen content of shoot and total green fodder yield. *Trichoderma* isolates were screened on the basis of fresh weight and dry weight of shoot and root. *Rhizobium* isolate R-1 and *T. harzianum* isolate TH-1 were found to be efficient. The cultural conditions of *Trichoderma harzianum* were standardized, pH range of 5.5-7.5 and temperatures of 25-30°C was found to be best for optimum growth of *T. harzianum*. Glucose and Ammonium Sulphate were better carbon and nitrogen sources to support the growth of *T. harzianum* and *T. viride*. The results of *in vitro* experiment showed maximum chitinase activity in TH-4 isolate. Early germination of Shaftal seeds was observed due to treatment with culture filtrate of all the four isolates of *T. harzianum* and an isolate of *T. viride*. Field trial was conducted using R-1, TH-1 and TV isolates (alone and in combination). Non-significant increase has been observed in green fodder yield of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 in the treatments of *Rhizobium* and *Trichoderma* spp. Number of substrates were tested for optimum growth of *T. harzianum* and *T. viride* and result indicated that wheat bran was the most suitable substrate followed by sugarcane bagasse for growth of *T. harzianum* and *T. viride*.

Key Words : *Rhizobium, Trichoderma harzianum, Trichoderma viride,* Nitrogen fixation

Signature of the major advisor **Signature of the student**

CONTENTS

Chapter - I

INTRODUCTION

Nitrogen fixation is the process by which the atmospheric nitrogen is reduced to ammonia. Nitrogen is an inert gas and only certain bacteria are capable of carrying out this process, the genus *Rhizobium* being the most efficient. The symbiotic association between *Rhizobium* and legumes has been reported to fix 24-584 kg N/ha annually in different legumes (Sindhu and Dadarwal 2000). So, this endosymbiotic association reduces the dependency of agriculture on fossil fuels derived nitrogen fertilizers. The high cost of fertilizers, rapid deletion of nonrenewable sources, release of pollutants during fertilizers production, disruption of nutrient balance in soil, leaching of nutrients in ground water and pollutants in surface water has emphasized the need of *Rhizobium* fertilizers to increase production of legume crops.

Symbiotic nitrogen fixation starts only after the formation of nodules, which is preceded by the colonization of rhizosphere and the infection of legume by specific rhizobia. Even presence of nodules on the roots of leguminous plants does not mean that nitrogen is being fixed as rhizobia can induce nodule formation but may fail to fix N_2 efficiently. Moreover, all the strains of a given *Rhizobium* spp may not be equally effective in increasing the yield of legume crops. Success of *Rhizobium* inoculation depends upon many factors that includes efficiency of *Rhizobium* strain, variety of leguminous crop, soil pH and soil

fertility (Deka and Kakati 1996) particularly N and P and competitiveness (Sindhu and Dadarwal 2000) and type of cultivar (Barhate *et al* 1999). Therefore screening of efficient strains of *Rhizobium* is important for maximum nitrogen fixation.

Trichoderma harzianum is another important organism occurring in the soil rhizosphere and well known for its potency to manage successfully the root diseases of crops. It is a free-living fungi that is highly interactive in roots and rhizosphere (Harman *et al* 2004). It can compete with the root exudates and remains in soil, multiplies, causes colonization of roots and always offer protection of plant root by improving the biological soil suppressiveness. But the survival and multiplication of *T. harzianum* is greatly effected by physical, chemical and biological factors in soil. Root colonization by *Trichoderma* spp also frequently enhances root growth and development, crop productivity and resistance to abiotic stress and uptake and use of nutrients. Moreover, it has been reported that besides acting as biocontrol agent, *Trichoderma* produces growth-promoting substances, which help in enhancing plant growth and productivity. These also induce resistance in plants (Bostock 2001; Bakker *et al* 2003 and Harman *et al* 2004).

Although *Rhizobium* and *T. harzianum* occurs together in the rhizosphere but not much information is available on their interaction, their effect on nodulation, nitrogen fixation and yield of a legume crop. Keeping in view, the little information available on the interaction and their effect on the host, the present study has been conducted on Shaftal (*Trifolium resupinatum* L.) variety

Sh-69. *Trifolium resupinatum* commonly known as *Shaftal, Chhattala or Bhukal* is highly nutritious leguminous fodder crop grown in northern India particularly in the irrigated areas of Punjab state. It contains high protein content (21%) than the other green fodders and more water content than the other green forage crops. It can tolerate adverse soil and climatic conditions. The present investigations was conducted with the following objectives:

1. Isolation, purification and screening of *Rhizobium leguminosarum* bv *trifolii* and *T. harzianum* from Shaftal (*Trifolium resupinatum* L.) variety Sh-69 and rhizosphere respectively.
2. Ecological studies of *Rhizobium leguminosarum* bv *trifolii* and *T. harzianum.*
3. Standardization of conditions like Temperature, pH, carbon and nitrogen sources for growth of *T. harzianum.*
4. Measurement of chitinase activity of *T. harzianum* involved in antagonistic action.
5. Effect of *Rhizobium leguminosarum* bv *trifolii* and *T. harzianum* and their interaction on nitrogen fixation and various growth characteristics of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under pot culture and field conditions.

Chapter - II

REVIEW OF LITERATURE

Hellriegal and Wilfarths (1888) discovered the ability of *Rhizobium* to fix atmospheric nitrogen. Beijerinick (1888) isolated nodule-forming bacteria and named it as *Bacillus radiocicola.* The natural contaminant of *Rhizobium* is *Agrobacterium radiobacter*. Bernaerts and Deley (1963) prescribed α– ketolactase test to differentiate between *Rhizobium* and *Agrobacterium*. Then Hahn (1966) prescribed a selective medium for isolation of *Rhizobium* by incorporating congo red (0.025 g/l) in Yeast extract mannitol agar (YEMA). Rhizobia are Gram negative, nonspore forming, aerobic rods, motile and have either one polar, subpolar or 2-6 peritrichous flagella. *Rhizobium* spp are differentiated on the basis of host plant that they infect and nodulate.

2.1 EFFECT OF *RHIZOBIUM* ON NODULATION AND NITROGEN FIXATION

Survey of old literature reveals that that there are number of reports regarding beneficial effect of *Rhizobium* on nodulation and nitrogen fixation. Coventry *et al* (1985) studied the development of population of *Rhizobium trifolii* and nodulation of subterranean clover. Soil samples were collected from about 28 sites following the various periods of cropping in a crop pasture rotation. *Rhizobium* population was less than 10^3/g of soil for 89 per cent of the sites. They found that application of lime resulted in a build up of *R.trifolii* in the absence of host legume. When inoculated clover seed were sown the population build up to

satisfactory levels. The number of nodules/ plant was increased by the application of lime.

Batra and Ghai (1988) studied the effect of soil salinity levels and inoculation of four forage legumes, viz Persian clover, Egyptian clover, lucerne and sweet clover under green house conditions. They found that Egyptian and Persian clover gave significantly greater green matter yield (258.6 and 259.5g/pot respectively) than lucerne (60.6g/pot). Inoculation of seeds with *Rhizobium* culture also increased the green matter yields of legumes from 164.8 to 204.8g/pot.

Friedericks *et al* (1990) evaluated African *Trifolium* sp for the growth and Biological Nitrogen fixation. *R. trifolii* strains were isolated from the two Ethiopian soils. These strains were examined for the symbiotic effectiveness on 5 African annual clover spp. They found that all the *R. trifolii* strains exhibited varying levels of symbiotic effectiveness. They were able to identify strains that were highly effective for each clover spp.

Kishinerisky *et al* (1992) conducted experiments on berseem variety Miscawi in the green fodder house using sterilized sandy soils under two sets of conditions- inoculated and uninoculated. Inoculation with the seven strains of *R. trifolii* produced the highest N accumulation. Inoculation increased the dry matter yield of *T. alexandrium* by 39 per cent in first cutting but had to effect three cuttings later.

Gok and Martin (1993) conducted an experiment to study the effect of *Rhizobium* inoculation on nitrogen fixation by soyabeans, clover and vetch

plants. They inoculated soyabean with 7 *Bradyrhizobium japonicum* strains and clover with 5 *R. leguminosarum* bv *trifolii* strains and vetch with 11 *R. leguminosarum* bv *viceae* strains. The inoculation of *Rhizobium* significantly increased the N_2 fixation and dry matter production in soyabean and clover but vetch showed no significant effect of inoculation.

Dubach and Ruselle (1994) studied the forage legume root and nodules and their effect on nitrogen transfer in alfalfa. They examined the amount of nitrogen in living and dead roots and nodules of alfalfa (*Medicago sativa* L.) and birdsfoot trefoil (*Lotus corniculatus* L.). Five roots contained up to 69 per cent of nitrogen from fixation in alfalfa and 49 per cent in the birdsfoot trefoil and nodules contained 9 per cent and 94 per cent fixed nitrogen respectively. They concluded that alfalfa releases more nitrogen than birdsfoot trefoil. Parco *et al* (1994) conducted an experiment on inoculation to determine if motility affect the ability of *Rhizobium* to nodulate and colonize the root system. Clover was inoculated with the motile and nonmotile strains of *Rhizobium trifolii*. They concluded that the plants inoculated with the motile strain were more nodulated as compared to the nonmotile strains of *Rhizobium*.

Pugh *et al* (1995) performed experiments with nodulated, pot grown plants of white clover (*Trifolium repens* L.) These were exposed to different soil moisture regimes and effect of these treatments was examined on dry matter production, nitrogenase activity and bacteroid distribution was determined. There was a marked decrease in nitrogenase activity when plants that had been

watered normally, suggesting the sensitivity of white clover to sudden changes in the moisture conditions.

Shukla and Dixit (1996) studied the response of *Phaseolus radiatus* L. to *Rhizobium* inoculation, plant population and phosphorus levels. They reported enhanced nodulation, nutrient uptake and yield due to *Rhizobium* inoculation.

Ibekwe *et al* (1997) examined the differentiation of clover *Rhizobium* isolated from biosolids – amended soils. Two phenotypic groups of effective and ineffective isolates were identified using the symbiotic effectiveness test. Effective nodules were associated with higher soil pH regardless of soil metal count. Isolates from most heavily contaminated soils were more variable than the isolates from control soils. They concluded that the soil pH was important in selection of rhizobia that formed ineffective N_2 fixing symbiosis. Patra and Bhattacharya (1997) studied the effect of *Rhizobium* on nitrogen fixation and yield of *Vigna radiata* cultivar B 1. They reported that the seeds treated with *Rhizobium* increased nodulation as compared to control.

Patra and Bhattacharya (1998) conducted a pot experiment to study the effects of *Rhizobium* (strain M10 of cowpea group) on seed inoculation, seedling inoculation or inoculum 15 days after sowing on *Vigna radiata* cv. B1 in unsterilized soil. They concluded that seed inoculated plants exhibited significantly greater shoot and root length and fresh and dry weights as compared to uninoculated control plants.

Maldal and Ray (1999) conducted an experiment to study the effect of *Rhizobium* inoculation and nitrogenous fertilizers on the performance of moong *(Vigna radiata)* cv. B105, B1 and Hooghly local. They concluded that seed treated with *Rhizobium* or 20, 30 and 40 kg N/ha showed greater nodulation with inoculation in B105 and Hooghly local gave the best overall performance.

Number of other workers has reported increase in nodulation and nitrogen fixation due to inoculation with *Rhizobium* strains in soyabean (Gao and Yang 1995; Rani and Kodandaramaiah 1997), blackgram (Prabhakaran and Ramaswamy 1990), chickpea (Khurana and Dudeja 1981), cowpea (Gregr 1990; Rajput and Singh 1996) and pea (Brewin *et al* 1993 and Feng *et al* 1997).

2.2 SCREENING OF EFFICIENT *RHIZOBIUM* STRAINS

In leguminous plants, symbiotic nitrogen fixation starts after the formation of nodules, which is preceded by the colonization of rhizosphere and the infection of legume by specific rhizobia. Even presence of nodules on the roots of leguminous plants does not mean that nitrogen is being fixed as rhizobia can induce nodule formation but may fail to fix N_2 efficiently. Moreover, all the strains of a given *Rhizobium* spp may not be equally effective in increasing the yield of legume crops. Success of *Rhizobium* inoculation depends upon many factors that includes efficiency of *Rhizobium* strain, variety of leguminous crop, soil pH and soil fertility (Deka and Kakati 1996) N and P, competitiveness (Sindhu and Dadarwal, 2000) and type of cultivar (Barhate *et al* 1999). This lead to an idea that screening of efficient strains of *Rhizobium* is required for maximum

nitrogen fixation.

Bhatnagar *et al* (1988) examined the Host-*Rhizobium* symbiotic interaction in mungbean (*Vigna radiata*). They reported that the inoculation of seeds with competitive and symbiotically effective rhizobia results in increase of N_2 fixation. They used three lines highly variable for nitrogen fixation characters and inoculated with 5 cowpea strains. They found that strain M3 was highly efficient in increasing nitrogen content with line 11152 but not with other lines. The strain CB756 was inefficient for all nitrogen fixation characters with all three lines. The combination of line 11152 and M3 gave the highest nitrogen content, dry weight/plant but the least number of nodules/ plant and the highest nodule dry weight.

Rasal *et al* (1989) conducted an experiment for the comparative study of five *Rhizobium* inoculants obtained from different sources. They reported that the total count of *Rhizobium* and effectiveness were responsible for the dry matter production and nodulation. In addition to the total viable cell count of the inoculant, variation in the effectiveness of rhizobial strains affected the number of nodules and plant dry weight. Herridge *et al* (1994) concluded similar results. They reported that the inoculation of legume seeds with competitive and symbiotically effective rhizobia results in increased N_2 fixation.

Gupta and Namedo (1996) studied the effect of different rhizobial strains on symbiotic traits and yield of the grains of chickpea (*Cicer arietinum* L.) cultivar JG 315. Out of 18 strains of *Rhizobium*, strain H60 was found to be the

most efficient in terms of highest grain yield. Kumar *et al* (1997) studied the response of *Rhizobium* strains for nitrogen fixation efficiency and biomass production in groundnut. *Rhizobium* strain NC-92 was efficient in comparison with other strains as it resulted in high number of nodules and dry weights of root and shoot.

Barhate *et al* (1999) conducted an experiment to study the interaction of 3 chickpea cultivars with 6 different strains of *Rhizobium*. The cultivar Vishal produced high nodule number and dry weight than the strain Vijay. The grain was reported to be maximum in the strain PG 93009.

Duodu *et al* (1999) reported the positive role of rhizobiotoxine in Legume-*Rhizobium* symbiosis in *Vigna radiata*. This rhizobiotoxine was produced by *Bradyrhizobium elkaii* and causes chlorosis in a variety of legume plants. They concluded that a plant hormone, ethylene that causes the inhibition in the nodule development was inhibited by this rhizobiotoxine.

2.3 EFFECT OF *RHIZOBIUM* ON DIFFERENT PARAMETERS

2.3.1 Leghaemoglobin and N_2 fixation

The nodules of legume plants contain soluble haemoglobin like pigment, which imparts a pink colour to the nodules and related to its O_2 consumption. is known as Leghaemoglobin (Kubo, 1939).Oxygenation cycle of soyabean leghaemoglobin was introduced by Keilen and Wang (1945). They observed that only spectroscopic ally detectable reaction in nodule was:

Ferrous leghaemoglobin + O_2 Lb> ferrous oxyleghaemoglobin (LbO_2)

Viratanen (1947) concluded that the leghaemoglobin content could be correlated with N_2 fixing capacity in pea and nodules of many other legumes. Smith (1949) conducted an experiment to show that leghaemoglobin is confined to the central tissue of nodules surrounding the bacteroids within the membrane envelope. Trouchet (1972) and Gourret and Fernandes (1979) have drawn the similar results in pea.

Earlier there was a controversy regarding the function of leghaemoglobin in symbiotic N_2 fixation. Scholander (1960) and Yocum (1964) had drawn a conclusion that leghaemoglobin function by allowing O_2 to penetrate the nodule at a very low level of free oxygen but at a sufficiently high rate to cope up with the energy requirement of N_2 fixation. Johanson and Homes (1973) concluded that there is high correlation between leghaemoglobin content of N_2 fixation, They observed that the rate of conversion of acetylene to ethylene /mg of leghaemoglobin constantly decreased during the growth season of soyabean whereas leghaemoglobin content/g fresh weight of nodules remained relatively constant. They compared the leghaemoglobin content of nodules with the rate of N_2 fixation in plants of soyabean cultivar at different stages of development.

Bergerson *et al* (1973) introduced the possible function of leghaemoglobin in the regulation of intranodule nitrate tension. Leghaemoglobin plays a dual role in N_2 fixation, first as carrier of O_2 to mitochondria and bacteroid for ATP generation via oxidative phosphorylation and second as modulator of oxygen tension to prevent inhibitory level of O_2 from reaching nitrogenase system.

Davidson (1973) studied the influence of rhizobial strain and soyabean variety on leghaemoglobin, nodule weight and N_2 fixation. He reported that the soyabean contain a characteristic leghaemoglobin.

Chahal and Rewari (1975) determined the relationship between the quantity of leghaemoglobin and efficiency of *Rhizobium* strains of mung and found that leghaemoglobin has a direct relationship with the efficiency of rhizobia and similar observations have been reported by many other workers (Bergerson, 1961 and Chopra and Subba Rao, 1967).

Huang and Chiying (1982) applied the higher levels of NH_4NO_3 (100mM) to soyabean plant 31 days after germination. They found that the nitrogenase activity was inhibited by NH_4NO_3 and this reduction was closely related to decrease in leghaemoglobin content. Dadson and Acquaah (1984) conducted an experiment to determine the effect of *Rhizobium japonicum*, N and P on nodulation, N_2 fixation and yield of soyabean. The treatment significantly increase plant height, number of pods/plant, leaf area index, total dry matter /plant, grain yield and seed yield. Low rates of N and P promoted nodule number, dry weight and leghaemoglobin content of nodules. Poi and Kabi (1982) conducted an experiment on winged bean and observed a substantial increase in nodule number, leghaemoglobin content of nodules over control.

Nelson (1987) evaluated the efficiency of short-term application of NH_4NO_3 on the nodule function. He concluded that acetylene reduction and leghaemoglobin content of nodules were reduced with the increasing

concentration of NH_4NO_3. Other growth parameters such as dry weight and nodule number were also affected in similar manner. Sairam *et al* (1989) studied the effect of P levels and effect of *Rhizobium* inoculation on nodulation, leghaemoglobin content and nitrogen uptake in fodder cowpea. *Rhizobium* inoculation and increasing P rates (0.90 kg/ha) increased Nodulation, leghaemoglobin content, N uptake and dry matter production in cowpea.

Xu *et al* (1989) studied the relationship between photosynthesis and N_2 fixation in symbiotic system of soybean and rhizobia. Field grown and hydroponically cultured inoculated plants had a higher N content, chlorophyll content and seed yield than the uninoculated control.

Lee *et al* (1990) reported that the *Rhizobium* inoculation increased nitrogen uptake, N content, chlorophyll content, total dry weight and number of pods/plant in groundnut. Posypanov *et al* (1990) studied the compatibility of red clover cultivar and *Rhizobium*. They inoculated the seeds with *Rhizobium* strains and reported the increased leghaemoglobin content, leaf area, chlorophyll contents, plant height, N and dry matter production.

Rai (1992) studied the effect of *Rhizobium* and nitrogen levels on nitrogen fixation and grain yield of *Phaseolus vulgaris*. Two rhizobial strains (ND1 and ND2) and two *P. vulgaris* cultivars (H137 and VL63) produced greater nodulation activity, plant N content and seed yield in saline-sodic soil, with 12.5 mgN, compared with other strains but interaction between cultivars and *Rhizobium* strains was significant in normal soils with 12.5mg N as compared with salt

tolerant strains.

Sangakkara (1993) studied the relationship between soil moisture, growth yield and N_2 fixation in selected grain legumes i.e. cowpea cv. M135, mungbean cv. M15 were sown in pots of 1:1 top soil and sieved river sand, soil moisture regimes with 20 per cent, 40 per cent, 60 per cent and 80 per cent depletion of the water content. They found nodulation and N_2 fixation levels were reduced in both species. It was concluded that under stress conditions cowpeas are more adaptable in terms of plant growth, yielding ability and N_2 fixation.

Singh *et al* (1994) conducted green house experiment on chickpea (*Cicer arietinum* L.) cv H-82-91 to study the metabolism in nodules during their natural and induced senescence. In natural senescencing nodules, maximum nitrogen activity was observed at 80-95 DAS linked with total soluble carbohydrates (TSC) and leghaemoglobin (Lb) content. NRA and NO_2 were found to be maximum at 100 DAS, whereas NO_2 and N remained up to 90 DAS. To induce the nodule senescence, KNO_3 (10mM and 20mM) and urea (5mM and 10mM) were applied separately at various growth stages. They found that during induced nodule senescence nitrogenase activity, Lb, TSC and nodule respiration were significantly decreased up to 10 days after treatment at all sampling stages.

Graham *et al* (2000) studied the perspective in N_2 fixation. They reported the decline in agricultural dependence on symbiotic N_2 fixation and use of rhizobial inoculants. Their review contrasts the potential contribution of biological fixed nitrogen, intensive and extensive agriculture systems and

opportunities for continued major contribution in N_2 fixation. They examined some opportunities for the improvement in N_2 fixation likely to arise through the advances in molecular biology.

2.3.2 Nitrate reductase activity and chlorophyll contents

Nitrate is the known source of N absorbed by the plants from soil. Nitrate uptake and its subsequent reduction by nitrate reductase is the primary path of nitrogen utilization in non-leguminous plants. In leguminous plants, the nitrogen requirement is met from the fixation of atmosphere through the symbiotic relation with *Rhizobium*. Chlorophyll is well known for increasing the yield of different crops but quantity may differ.

Brougham (1960) studied the relationship between the critical leaf area, total chlorophyll content and maximum growth rate of some pasture and crop plants. There was a highly significant correlation (r = +0.912) between the maximum growth rates of different species and amount of chlorophyll above the measured level/ unit area of land. However, the index is equal to the growth rate/ total chlorophyll was higher for species in which leaves were disposed horizontally or where flagging occurred (clover, maize etc) than for the grass species.

Enzymes involved i.e. nitrate and nitrite reductases are important for regulation and associated with the process of nitrate assimilation and seed protein accumulation in crop plants. Broughton *et al* (1978) concluded that the nitrate reductase in the leguminous plants is detected in roots and it supplies the nitrogen

till nitrogen fixation starts. Skrdleta *et al* (1980) studied the effect of nitrate on nitrogenase activity, and growth of nodule tissue in pea plants (*Pisum sativum* L.). There was a significant decrease in nitrogenase activity after the application of nitrate. However, the addition of nitrate leads to its rapid accumulation in the nodule and leaf tissue, which simultaneously induce nitrate reductase activity.

Sharma and Chahal (1983) reported the significant increase in the dry matter, yield and chlorophyll content of lentil (*Lens esculenta* L.) due to Mo application and *Rhizobium* inoculation. Terry (1983) indicated that reduction in chlorophyll content of Fe stressed plants was accomplished by reduction in electron transport component as a per unit area basis. He observed that in Fe stressed leaves the ultrastructure of chloroplasts was changed but mitochondria and microbodies were unchanged. Thus they concluded that the Fe play a very important role in the development and maintenance of photosynthetically active tissue (Pushnik *et al* 1984).

Sawhney *et al* (1985) reported that the application of nitrate resulted in retardation of nodule development and exerted a delay effect on rate of nitrogen fixation. They observed enhanced nitrate reductase activity in leaves as well as in the nodules. They found that application of nitrate induced the premature senescence of plants and lowered the number and weight of seeds.

Mahadkar and Saraf (1988) studied the nitrate reductase activity in relation to nodulation and nitrogen fixation in *Vigna radiata*. They reported that seed inoculation with *Rhizobium* was most effective in increasing nodulation.

Nitrate reductase activity was high 15 days after sowing (DAS), lowest 30 DAS and highest 45 DAS and thereafter decreased gradually. Higher enzyme activity during the flowering period and prior to termination of nodulation indicated the nitrogen source to be nitrate from the soil.

Caba *et al* (1990) studied the nitrate reductase and nitrite reductase activity in roots and nodules of faba beans. These were grown in the presence of nitrogen alone or with additional nitrate in the medium. They found that nitrate reductase enzymes were stimulated by addition of nitrate while nitrate reductase activity or little nitrite reductase activity was found for nodules of faba beans.

Chahal and Sharma (1991) showed the relative behaviour of 4 chickpea cultivars (G130, C235, GL769 and G543) for nitrogen fixation and utilization of nitrate. NRA of leaves was measured at 25, 50 and 75 days. The cultivar G130 showed the maximum NRA at initial stages which decreasing subsequently. They found a negative correlation between the NRA and nitrogenase activity. Hervas *et al* (1991) studied growth, nitrate reductase activity (NRA), nitrogen content and soluble protein concentration in pea (*Pisum sativum* cv *lineolin*). Plant supplied with different NO^-_3 concentration and inoculation with *Rhizobium leguminosarum* strain. It has been observed that difference in tissue NRA was mainly related to NO^-_3 concentration in growth medium. However, nodulation markedly influence the amounts of NRA in different plant organs specially root. The concentration of NO^-_3 in leaves, stems and roots increased with NO^-_3 dose. They observed that leaf and stem area was not significantly affected by

nodulation.

Batra *et al* (1992) studied the effect of nitrate application on nodulation and growth of Rajmaah (*Phaseolus vulgaris* cv VL-63). These were grown in the pots filled with 1 kg of sterilized soil mixed with 20g FYM and 30 mg P_2O_5 and given 0, 10, 20, 30, 40, 50ppm N as KNO_3 with and without inoculation of *Rhizobium* isolates. They observed that the dry weight of shoot was 1.99-2.98 and 1.31-2.94g/pot in inoculated and uninoculated plants respectively. Furlani *et al* (1996) conducted a green house experiment using six N levels (28,56,84,112,140 and 168mg/l of N) to adjust the chlorophyll reading meter to the nitrogen levels in case of beans. Numbers of parameters viz. chlorophyll content, leaf area, Ca, Mg concentration, grain yield, fresh and dry matter of leaves were evaluated.

2.4 COMPETITIVENESS

Competitiveness of given *Rhizobium* strains is the ability to make the nodules and to fix nitrogen in an environment where plants are exposed to other strains of *Rhizobium*. Number of techniques has been used in the past to study the competitiveness of rhizobia i.e. serological techniques (Jimbo 1930; Vincent 1963 and Skrdleta and Karimora 1961) and genetic markers for antibiotic resistance (Schwinghamer and Dadman 1973).

The effectivity of given *Rhizobium* strain is not same in field and laboratory. Roughly *et al* (1976) concluded that the introduction of efficient strain of *R. trifolii* showed better results than the natural strains under field conditions

but the persistence of introduced strain in subsequent years decreased. This indicates the poor survival and compatibility of the introduced strain.

Number of other workers conducted the similar work (Johnston and Beringer 1976; Boonkerd *et al* 1978; Amarger and Lobreau 1982; Mcloughlin *et al* 1984; Josephson and Pepper 1984; Renwick and Jones 1985; Mcloughlin and Dunican 1985 and Valdivia *et al* 1988 and Yaman 1995).

Cooper *et al* (1990) studied the competitiveness between *Rhizobium* strains for the infection and nodulation of legumes. *Rhizobium trifolii* varied in speed of entering the no root hair zone of white clover (*Trifolium resupinatum*). He observed a positive correlation between speed and nodulation competitiveness in mixed culture. Fabiano and Arias (1991) studied competitiveness between the native *Rhizobium leguminosarum* bv *trifolii* and 2 commercial strains (U28 and WU290) for nodulation in clover. The ratio between the number of nodules containing the naturalized isolate (P4B) and commercialized strain (U28) were directly proportional to their respective inoculum densities, when white clover was used as host plant. However, when P4B and WU290 were used in different mixtures, the results suggested that the naturalized strain was useful in maintaining the high yield.

Thies *et al* (1992) observed that the size of the individual rhizobial strain was the most significant in affecting the success of inoculation. This effect has been studied I case of pea, groundnut and clover. They gave an equation $Y=97.88 - 15.03 (\log 10X^{+1})$ that describes that X is the most probable number of

indigenous rhizobia /g of soil.

Athar and Doughlas (1996) examined 20 strains of *R. melilotii* for their competitive ability with a streptomycin resistant mutant of commercially available strain 102F519. The seedlings of alfalfa (*Medicago sativa* L. and *Medicago falcata* L.) were grown in agar medium tubes inoculated with 20 rhizobial strains and equal proportion of streptomycin resistant strain. They concluded that the inoculated plant had greater dry weights and 10 rhizobial strains showed greater nodule occupancy than the plant inoculated with the commercial streptomycin resistant strain. Butivana *et al* (1997) studied the competitiveness in *R. melilotii*, *R. leguminosarum* and *Bradyrhizobium japonicum* strains. They reported that all the strains were more effective in field.

Sindhu and Dadarwal (2000) reviewed that competitiveness among rhizobial strains in the rhizosphere to dominate nodulation of legume roots is a complex process and it involves the interaction of biotic and abiotic components of soil ecosystem. The identification of efficient and competitive strains of rhizobia for different legume crops and an understanding of the molecular and genetic basis of competitiveness will allow genetic manipulation for enhanced nodulation. They concluded an alternative approach could be employed which involve the alteration of host cultivars through breeding program that can restrict the nodulation by the indigenous competitive strains and allow nodulation only by the inoculated strains.

2.5 ISOLATION, PURIFICATION AND CHARACTERIZATION OF *TRICHODERMA HARZIANUM*

Trichoderma spp. are most prevalent culturable fungi present nearly in all soils and other diverse habitats. Persoon (Grondona *et al* 1997) introduced the genus *Trichoderma* about 200 years ago. Due to rapid growth of soil fungi it is very difficult to estimate the population of *Trichoderma* in soil.

Trichoderma is a saprophytic fungi and can be grown on the artificial medium prescribed by Martin (1950). He used an acid; rose bengal and streptomycin for estimation of soil fungi by plate count method. Later, a selective medium was developed by Tsao (1970) for the isolation of pathogenic fungi. But it has been observed that some *Trichoderma* isolates grow faster on Martin medium as compared to selective medium.

For quantitative estimation, *Trichoderma*-selective agar medium (TSM) was developed by Elad *et al* (1981). In this medium, selectivity was obtained by incorporation of chloramphenicol as a bacterial inhibitor and rose-bengal as selective fungal inhibitors. TSM also contains the low concentration of glucose, which still allows relatively rapid growth, and sporulation of *Trichoderma* and rapid identification of *Trichoderma* spp.

During the isolation of *Trichoderma,* in past, the scientists faced number of problems. One of the major problems was that fungi were fast growing, so these fungal colonies had to be marked everyday and in the case of fast growing fungus, these had to be transferred immediately to the other plate. To overcome

this problem, if soil samples were diluted for the quantitative estimation of *Trichoderma spp*, Soil species belong to fungi imperfecti often grow faster and ultimately prevent the growth of *Trichoderma* spp. These problems were faced when some scientists (Smith and Dowson, 1944) count *Trichoderma* spp. in soil used soil extract agar supplemented with rose-bengal. Gindrat and Ricard (1976) developed the counting techniques for *Trichoderma viride* conidia dispersed in barley flour inoculant.

For the mass production of *Trichoderma*, agricultural byproducts are the suitable media. In the past number of workers have used these byproducts (Das *et al* 1997; Hari and Somasekhar 1998; Jayaraj and Ramabadran 1996, Prakash *et al* 1999 and Shamarao *et al* 1998; Gandhikumar *et al* 2001).

Jayaraj and Ramabadran (1996) conducted an experiment with cheaper organic substrates to evaluate the mass multiplication of *T. harzianum* with and without nutrient additives. Out of 7 organic substrates tested pigeon pea husk was the best followed by starch extract and press mud. Addition of urea reduced the multiplication, whereas ammonium sulfate enhanced the colonization and propagule number of *T. harzianum*.

Das *et al* (1997) used five media (wheat bran, rice bran, maize meal, sand media, potato dextrose agar and sawdust) for examining the mass production of *Trichoderma* spp. Wheat bran was proved to be more promising for the growth and sporulation of the fungi and significantly higher after 14 days than after 7 days of incubation.

Hari and Somasekhar (1998) used sugarcane byproducts (bagasse, trash and pressmud) for the mass multiplication of *Trichoderma* spp. They concluded that the best growth of *Trichoderma* occurred on bagasse.

Gandhikumar *et al* (2001) used various carriers (black gram shell powder, shelled maize cob powder, coir pith, gypsum, peat and talc) to evaluate the growth of *Trichoderma* spp. They reported the highest colony forming units (CFU) of *T. harzianum* and *T. viride* on cob maize powder followed by black gram shell powder. Peat recorded the lowest number of CFU of both *Trichoderma* species after 30 days of storage.

2.5.1 Effect of different parameters on growth of *Trichoderma* spp.

Trichoderma is a potent biocontrol agent and used in number of experiments but sometimes it gives inconsistent results. This is due to the reason that less information is available on the nutritional requirement of *Trichoderma.* As specific nutrients affects the production of antifungal metabolite (Aube and Gagnon 1969; Danielson and Davey 1973 a, b; Jackson *et al* 1991; Saha and Pan 1998; Altomare *et al* 1999 and Monga 2001).

Ahmad & Baker (1987) conducted an experiment to study the growth of *Trichoderma harzianum* mutants on Saccharose, cellulose and xylan as a carbon source. Mutant strains significantly produced higher biomass than the rhizosphere competent wild types. They reported that the ability of mutants to grow rapidly on complex carbon sources than their wild type and to increase biomass could be of ecological significance and a characteristic of a rhizosphere-

competence.

Altomare *et al* (1999) studied the capability of plant growth promoting fungi *Trichoderma harzianum* Rifai 1295-22, T-22 to solubilize minerals via three possible mechanisms i.e. acidification of the medium, production of the chelating metabolites and redox activity.

Monga (2001) conducted an experiment on nutritional requirements of *Trichoderma* and *Gliocladium* and reported that glucose as carbon source significantly affected the sporulation and growth of *Trichoderma* strains. He observed poor sporulation in *Trichoderma* with all carbon sources (glucose, fructose, maltose sucrose). However he observed excellent sporulation in *Gliocladium* with all carbon sources except maltose. He reported Potassium nitrate as the best source in case of *T. harzianum* (dry weight 309 mg/ 100 ml broth) followed by Ammonium chloride (dry weight 146 mg). In case of *T. viride,* dry weight was 249 mg by using potassium nitrate and 235 mg by using Ammonium chloride.

Temperature and pH of the medium greatly affected the growth and sporulation of *Trichoderma.* Jackson *et al* (1991) conducted an experiment to study the effect of temperature, pH and water potential on biomass production or hyphal extension of 3 *Trichoderma* isolates *in vitro.* They reported that optimum biomass production occurred at between 20 and 30°C and at pH range between 4.6 and 6.8. No isolate was able to grow at 40°C.

Malathrakis *et al* (1992) investigated the effect of substrate,

temperature and time of application on the antagonism of *Trichoderma sp* using *Botrytis cinerea*. They reported, earlier the application of the antagonists and the higher the incubation temp the stronger was the inhibition of *Botrytis cinerea*.

Fruzynska and Manka (1994) conducted an experiment to study the effect of 2 *Trichoderma s*pp (10 isolates) and 3 *Penicillium* spp. (isolates) on the growth of *Fusarium oxysporum* f. spp. *dianthis*. They reported that changing the pH of the medium from 6.5 to 4 or adding wheat grain or peat extracts to the medium did not influence growth. Incubation temperature with in the range of 10°C to 35°C also had no effect.

Larenas and Montealegre (1996) studied the effect of 2 storage temperature 6 and 22°C on the viability of *T. harzianum* pellets prepared with sodium alginate and wheat flour. The effect of the nutrient level on mycelial growth and development was assessed for 2 types of pellets (with 3 and 9% wheat flour). No difference was found between the 2 temperatures during 5 months of study; after 2 years of storage at room temperature, pellet germination was 89 per cent. The density in pellets germination was 89 per cent. The density in pellets with 95 per cent wheat flour was significantly greater than that with 3 per cent of flour. But in both the cases, mycelia showed the same development.

Cliquet and Scheffer (1997) studied the influence of culture conditions on growth and survival of conidia of *Trichoderma* spp. Coated on seeds through methylcellulose coating and an industrial film coating process. The percentage viability was between 23 and 44 per cent after methyl cellulose coating

stromaticum. The optimum growth and sporulation occurred at 20 and 30°C. No growth occurred at 10 and 35°C. Biomass production was maximum between pH 5.5 and 7.5 but growth occurred over whole range of pH values. Potato molasses recorded the highest conidia production. The highest dry weight yield was recorded from starch-maize steep liquor. In glucose-maize steep liquor the yield was high but sporulation did not occur.

2.6 BENEFICIAL EFFECTS OF *TRICHODERMA*

2.6.1 Biocontrol

Trichoderma is an efficient biocontrol agent. It shows the activity against number of pathogens i.e. *Rhizoctonia, Pythium* and *Sclerotium* etc. This antagonistic activity of *Trichoderma* against different fungi was reported by Dennis and Webster (1971a,b). They conducted an experiment to test the ability of number of isolates of *Trichoderma* to coil around the hyphae of other fungi belonging to basidiomycetes, zygomycetes, ascomycetes, oomycetes and several other fungi imperfecti.

The microorganisms growing in the rhizosphere are ideal for use as biocontrol agents since rhizosphere provides host line defence for roots against attack by pathogen. The mechanisms suggested to be involved in biocontrol are antibiosis, lysis, competition, mycoparasitism and promotion of plant growth (Harman *et al* 2004). It seems reasonable to assume that the successful antagonism may rely on the combination of these modes of action. Dennis and Webster (1971 a,b) first of all describe the antagonistic activities of *Trichoderma* with antibiotic

production. They elucidated that *Trichoderma* spp. produces volatile and nonvolatile compounds, which are capable of inhibiting the growth of variety of fungi. Dennis and Webster (1971) noted that active isolates were associated with the coconut smell. The substance responsible for this smell has been identified as 6-n-pentyl-2H-pyran-2-one (6PP) and its biological activity against the number of plant pathogens has been demonstrated (Ghisalberti and Sivasithamparam 1991).

Parasitism is a complex process in which *Trichoderma* shows growth chemotropically towards its host and then it attaches itself around the host hyphae. After attachment, it is penetrated and degrades the host cell wall. It has been found that local cell wall lysis occurs at a contact between host and antagonist (Cherif and Benhamou 1990). During the interaction of *Trichoderma* with the pathogen i.e. *Sclerotium* rolfsii and *Rhizoctonia solani,* the cell walls are enzymatically digested by the parasites. The number of scientists conducted the work on parasitism.

Mycoparasitism, where by *Trichoderma* attack other fungi by excreting the lytic enzymes as proteases, glucanases and chitinases (Elad *et al* 1982; Chet 1990; Cruz *et al* 1992; 1993; 1995 and Limon *et al* 1999). Elad *et al* (1982) showed that *Trichoderma harzianum* is responsible for the production of extracellular lytic enzyme that are directly involved in cell wall degradation of *R.solani* and *S. rolfsii*. The members of oomycetes like *Pythium* spp. also contain cellulase. *T. harzianum* excretes the enzymes like chitinases, β1, 3- glucanases if grown on chitin, laminarin and cell wall of *S. rolfsii* as the sole source of carbon.

Chitinase was excreted by *T. harzianum* when chitin served as sole carbon source, chitinase and β1,3-glucanases are the key enzymes in the lysis of fungal cell wall. The lytic extracellular enzymes are capable of degrading *S. rolfsii, R. solani* and *Pythium aphanidermatum* cell wall. Cruz *et al* (1995) showed that a novel endo β1,3- glucanase, BGN 13.1 is involved in mycoparasitism of *T.harzianum*. The mycoparasitism produces 3 extracellular β1, 3-glucanases and most of these named as BGN 13.1 was expressed when either cell wall polymers or autoclaved mycelium were used as the carbon source. The experimental evidence showed that it lyse yeast and other fungal cell wall.

Haran *et al* (1996) studied molecular mechanisms of lytic enzymes involved in the biocontrol activity. They concluded that there was initiation of mycoparasitic responses, consisting of morphological as well as biochemical changes, coiling induction of unique combinations of chitinolytic enzymes and induction of other cell-wall hydrolyzing enzymes.

Chitinolytic system of *T. harzianum* consists of five distinct enzymes depending upon the strain. *Trichoderma* isolate (isolate TM), the system composed of two 1,4-N-acetylglucosamine (102 and 73 kDa) and four endochitinases (52, 42, 33 and 31 kDa). These findings have been given by number of scientists (Lorito 1998 and Carsolio 1999).

Lorito (1998) elucidated that different components of chitinolytic system of *T. harzianum* probably involved in complementary modes of action of component enzymes. However, the entire system might be required for maximum

efficacy. The most interesting enzyme of the complex is the 42 kDa endochitinase (ech 42) which can be hydrolysed *in vitro Botrytis cinerea* cell walls and inhibits spore germination and germ tube elongation of various fungi. The ech 42 is strongly induced during fungus- fungus interaction and when the fungus is grown in presence of Autoclaved mycelia of several fungi or with colloidal chitin as sole carbon source (Carsolio 1999).

The induction of resistance in plants by *Trichoderma spp* has been studied. Localized and induced resistance occurs in response to attack by pathogenic microorganisms, physical damage due to insects on other factors, treatment with various chemical inducers and presence of non-pathogenic Rhizobacteria (Kuc 2001 and Oostendorp *et al* 2001).

First of all induced resistance in plants by *Trichoderma spp* was published by Bigirimana *et al* (1997). They showed that treating soil with *Trichoderma harzianum* T-39 made leaves of bean plants resistance to disease that are caused by fungal pathogens i.e. *B. cinerea* and *Colletotrichum lindimuthianum.* Though T-39 was present only on the roots and not on the foliage. De Meyer *et al* (1998) gave the same findings in other Dicotyledons plants. Similar studies have been carried out with a wide range of plants including Monocotyledons and Dicotyledons. But still much more remains to be understood about specific system involved.

2.6.2 Plant –*Trichoderma* Interaction

Trichoderma are well known for their ability to colonize roots.

Trichoderma conidia have also been applied to fruits, flowers and foliage and plants disease can be controlled by their application to any of these sites (Elad 1994 and Harman 2000). Some *Trichoderma* strains can colonize only local sites on roots (Metchell and Wilson 2001) but rhizosphere competent strains colonize entire root surfaces for several weeks (Thrane *et al* 1997) or months (Harman, 2001). In some cases *Trichoderma* hyphae invade the root epidermis and this invasion is usually limited to first or second layers of cells (Metchell and Wilson 2001; Yedida *et al* 1999; 2000).

Lo *et al*(1999)observed that some *Trichoderma* grow on leaf surfaces also. T-22 that expresses β-glucuronidase was applied through roots or the soil application did not yield leaf colonization. But after foliar spray application of conidia, the spores germinated and various T-22 hyphae developed. Thus *Trichoderma* strains can colonize leaf surfaces under certain conditions.

Yedida *et al* (2004) observed in cucumber and cotton that *Trichoderma* induces the plants to produce defence-related plant enzymes including various Peroxidases, chitinases, β1-3 glucanases etc. In cucumber, root colonization by strain T-203 causes an increase in the production of phenolic glucoside levels in leaves, which are strongly inhibitory to a range of bacteria and fungi. Thus, *Trichoderma* not only produce antibiotic substances directly, but they also strongly stimulate plants to produce their own antimicrobial compounds. Moreover, root colonization by *Trichoderma* results in significant changes in plant metabolic machinery. Proteomes studies from either treated or untreated with T-22

were fractioned by gel electrophoresis. It has been observed that about 40 per cent of the Proteins that were seen in the presence of T-22 were not observed in the untreated plants. *Trichoderma* spp. enhance plant growth and productivity. These also induce resistance in plants (Bostock 2001 and Bakker *et al* 2003). This enhanced plant growth resulting from amendments of soil with *Trichoderma harzianum* and *T. koningii* was investigated and it was found that increased growth could be attributed to the direct effect of these *Trichoderma spp* on the plant or a secondary effect due to control of minor plant pathogens. This potential has been seen in case of number floricultural and horticultural crops was determined by various workers (Wu 1982; Wintham *et al* 1986; Kleifeld and Chet 1992; Hyakumachi 1994; Cassiolato *et al* 1996; Bell *et al* 2000; Borregaard 2000; Bostock 2001; Bakker *et al* 2003; Heemert *et al* 2000 and Harman *et al* 2004).

Wu (1982) concluded that seeds treated with *Trichoderma pseudokoningii*-4 and 5 per cent methylcellulose suspension for 30 minutes showed significantly better results in the emergence of seeds than the untreated seeds. Similarly, Chang *et al* (1986) recorded increased plant growth with *T. harzianum*. They concluded that in raw soil containing the fungus, pepper seeds germinated 2 days earlier than the untreated control. Raw soil infested with *T. harzianum* hastened the flowering, increased the number of blooms/plant and increased height and weight of plants. Likewise, Harman *et al* (1989) conducted an experiment to study the effect of *Trichoderma harzianum* strains on seed treatments of cotton, cucumber, pea, snapbean, maize and wheat. They concluded

that strain T 12 of *T. harzianum* increased plant stand, reduced seedling mortality and increased plant growth as compared to the untreated plants. The increased plant growth was evident for the entire duration of trial. Kleifeld and Chet (1992) studied the interaction of *Trichoderma harzianum* with plants. They concluded that when *T. harzianum* was applied to pathogen free soil, increased emergence of seedlings, plant height, leaf area and dry weight of plants such as moongbean, radish, tomato, chilli and cucumber.

Hyakumachi (1994) isolated plant growth promoting fungi (PGPF) from the rhizosphere of *Zoysia tenuifolia,* wheat, maize and aubergines. He concluded that the frequency of occurrence of PGPF out of total fungi isolated from *Z. tenuifolia,* wheat, maize and aubergines were 46, 47, 37.9, 10 per cent respectively. The most efficient PGPF isolated from the rhizosphere of the 32 isolates screened, 14 belonged to the sterile group, 9 were *Trichoderma*, 5 were *fusarium,* 3 were *Penicillium* and 1 was *Mucor* isolate. Similarly, Shivana *et al* (1994) conducted an experiment with wheat cv. Kitami 53 and 54 gou. They concluded that 11 out of 18 sterile fungal isolates *Penicillium* and *Trichoderma* spp. increased shoot length, dry weights and induced the plants to produce long spikes and more seeds.

Michalikova (1995) studied the influence of biopreparation 'Trichonitrin' on winter wheat. He found that wheat grains soaked in Trichonitrin (a microbial pesticide obtained from *Trichoderma harzianum* strain B1) showed no signs of *Fusarium culmorum* infection but increased growth.

Skrobakova (1995) conducted an experiment with application of growth regulators and growth stimulators. The growth regulators were 'Supresivit' (microbial preparation obtained from *Trichoderma harzianum*) and growth regulators were rastim 30 DKV, Bio-S and Kadostime. He found that the yield increased with growth regulators was in the range of 3.7-5.2 per cent . Supresivit applied to seeds before sowing improved emergence as compared with untreated control.

Shivana *et al* (1996) isolated *Penicillium, Trichoderma* and some nonsporulating fungi from Zoysiagrass rhizosphere. They tested the ability of these fungi to enhance plant growth and found that the addition of fresh potting medium appeared to increase their growth promoting ability. It showed that fungi in soil might depend upon the availability of organic substrates as evidenced by the promotion of plant growth. Growth enhancement of Shrunken-2 (Sh 2) sweet corn by *T. harzianum* 1295-22 was reported by Bjorkman (1998).

Altomare and Norvell (1999) studied the capability of plant growth promoting fungi *Trichoderma harzianum* Rifai 1295-22, T-22 to solubilize minerals via three possible mechanisms i.e. acidification of the medium, production of the chelating metabolites and redox activity. Gonzalez *et al* (1999) studied the effect of *Trichoderma harzianum* on bacteria, fungi, actinomycetes and influence on growth of tomatoes and potatoes. They observed stimulatory effect on plant growth as fresh weight, plant height and diameter of stem was increased.

Bae *et al* (1995) studied the response of cucumber with the

amendments of the culture filtrates with *T. harzianum* T95 and *Gliocladium virens* G 872 and G 872B. They reported enhancement in the fresh weight and primary leaf area of cucumber cotyledons. Harman (2000) conducted an experiment to show the increase in the root development in maize and numerous other plants. He demonstrated that this effect could be induced by the addition of small amounts of fungus applied as seed treatment. The presence of root colonizing *Trichoderma* induced about as many deep roots intercepts 25-75 cm below the soil surface. This resulted in increased drought tolerance and resistance to compacted soils. The ability of *Trichoderma* to induce increased root formation is not restricted to maize or greenhouse crops and other growth characters of these plants can be enhanced by the presence of other root- colonizing microorganisms.

Bell *et al* (2000) studied the response of cucumber seedlings to inoculation with 7 isolates of *Trichoderma harzianum*. *Trichoderma* mixture was applied by three different methods; spore coated organic pellets, dried biomass powder or seed coating. They concluded that the number of healthy seedlings were less in the untreated field soil.

Naseby *et al* (2000) studied the effect of five strains of *Trichoderma* on plant growth. They concluded that *Trichoderma* strain N47 significantly increased pea fresh shoot weight by 15 per cent while strain T4 and N47 significantly increased root weights by 22 and 8 per cent respectively. Borregaard (2000) studied the effect of 'Supresivit' on the growth of *Lepidium sativum* and observed increased biomass of plants raised from seeds treated with

'Supresivit' (5g/100g seed). Two strains of *Trichoderma harzianum* TRI002 and TRI003 were introduced as granules and wettable powder respectively were introduced by Heemert *et al* (2000). As plant growth promoter, *T. harzianum* increased development of root system in several crops resulted in greater vitality, productivity and quality.

Whipps (2001) conducted an experiment with mixture of root colonizing biocontrol agents including *Trichoderma harzianum*. He concluded that mixture of biocontrol agents showed better results than its own. This ability has been examined in managed and natural ecosystems. Synergy has also been observed in mycorrhizal fungi and T-22 (Datnoff *et al* 1995 and Nemec *et al* 1996).

Generally, it is impossible to separate the direct effects on the plant growth from the control of pathogenic or other deleterious microorganisms that reduce plant growth. As in the interaction between T-22 and plant root and shoot growth increased in both sterilized and nonsterilized soil in the presence of soil fungicides. Harman *et al* (2004) showed improvement in root development are frequently associated with increase in yield and biomass. There are more than 500 commercial and academic trials to study the effects of T-22 on maize and the average increase in yield over those obtained using typical agricultural practices was 5 per cent.

Harman *et al* (2004) concluded that in most cases, improved root development and increase in plant growth are probably caused by biocontrol and

related effects on root-associated microflora, and by direct improvement of plant growth. Deleterious root microflora can reduce plant growth in the absence of plant disease. Some harmful root associated microflora produce cyanide to maintain their niche in the competition. *Trichoderma spp* are resistance to cyanide and produce two different enzymes that are capable of degrading it in the root zone (Ezzi and Lynch 2002). Therefore these fungi can directly increase the root growth, control deleterious nonpathogenic root microflora, destroy toxic metabolites produced by deleterious micro flora and directly control root pathogens. So, several different routes accomplish the enhancement of root growth with the improvement in the plant growth and resistance to stress. Each of these involves the multiple mechanisms as has already been described for the biological control of plant pathogens on roots and foliage.

2.6.3 *Rhizobium-Trichoderma* interaction

Rhizobium-Trichoderma interaction has been investigated *in vitro* and in field by number of workers. First of all Harman *et al* (1981) observed increase number of nodules on roots of plants grown from seeds treated with *Rhizobium* alone and in combination with *Rhizobium* and *Trichoderma*. Kehri and Chandra (1991) observed significant increase in the weight of nodules of moongbean plants raised from *T. viride* treated seeds. Kastov (1996) conducted an experiment with *Rhizobium* and *Bradyrhizobium* grown on composted paper sludge. They incubated this mixture at room temperature (22-24°C) for 5 days and added NP (1%W/W) and *Trichoderma harzianum*. They concluded that number of

Chapter- III

MATERIALS AND METHODS

The present investigation was carried out to study interaction of *Rhizobium leguminosarum* bv. *trifolii* and *Trichoderma harzianum* on nitrogen fixation in Shaftal (*Trifolium resupinatum* L.) variety Sh-69.

3.1 CULTURES

Cultures	Source of procurement
Rhizobium leguminosarum bv. *trifolii*	Isolated from nodules of Shaftal (*Trifolium resupinatum* L.).
Trichoderma harzianum	Isolated from rhizospheric soil of Shaftal (*Trifolium resupinatum* L.).
Trichoderma viride	Procured from Department of Plant Pathology, Punjab Agricultural University, Ludhiana.

3.1.1 Seeds

The seeds of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 were procured from Department of Plant Breeding, Punjab Agricultural University, Ludhiana.

3.1.2 *Rhizobium* isolates

Rhizobium leguminosarum bv *trifolii* isolates were obtained from the nodules of Shaftal plants collected from different places. Shaftal plants were

uprooted with root system intact. Healthy and pink nodules were selected with a portion of 3-4 mm of roots attached to it. These nodules were thoroughly washed to remove soil particles. The nodules were surface sterilized in 0.1 per cent $HgCl_2$ (acidified) solution for 2-5 minutes and then dipped in 95 per cent alcohol for 2 minutes. These nodules were crushed with the help of sterilized glass rod in sterilized water blanks. The suspension of crushed nodules was poured in to sterilized Petriplates (1ml in each Petriplate). Then YEMA medium (15-20ml) containing congo red was poured in to these plates and rotated clockwise and anticlockwise. After solidification, these plates were incubated at 28±°1C for 5 days. After incubation colonies of *Rhizobium* appeared on plates. These colonies were watery, translucent, shining, raised and comparatively small with entire margins in contrast to stained colonies of *Agrobacterium* on congo red medium. The colonies of *Agrobacterium* spp. appeared red. In total ten isolates of *Rhizobium leguminosarum* bv. *trifolii* were obtained which were further purified and characterized.

3.1.3 Purification

Ten isolates of *Rhizobium leguminosarum* bv *trifolii* were purified on YEMA medium with Congo red by streak plate method. After incubation, single colony was transferred to YEMA slants for further investigation. Ten isolates were obtained, purified, characterized and maintained on YEMA slants in refrigerator (4°C) for further studies.

Composition of YEMA medium

Ingredients	g/l
Mannitol	10.0
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Yeast extract	1.0
$CaCO_3$	3.0
Agar	20.0
Distilled water	1000 ml
pH	7.0

Congo red Yeast extract mannitol agar (CRYEMA) medium was prepared by adding 2.5ml of 1 per cent Congo red was added in to one litre of YEMA medium before sterilization. This medium was sterilized by autoclaving at 15 psi pressure (121°C) for 20 minutes.

a. Gram staining

Gram staining was performed to check the purity of *Rhizobium leguminosarum* bv *trifolii* isolates and to insure the absence of any Gram positive contaminant and *Agrobacterium*.

b. Ketolactose test (Bernaerts and Deley 1963)

The test ensures differentiation between *Rhizobium leguminosarum* bv *trifolii* and *Agrobacterium* species produce an enzyme, ketolactase, which convert lactose to ketolactose whereas *Rhizobium leguminosarum* bv *trifolii* do not. It can reduce Benedict's reagent to form yellow coloured zone of Cu_2O around colonies.

Petriplates containing lactose agar medium were inoculated with isolates of all the *Rhizobium leguminosarum* bv *trifolii*. These plates were incubated at 28±1°C for 5 days. Then the Benedict's reagent was poured in to Petriplates and kept at room temperature for about one hour. The absence of yellow colored zone of Cu_2O around colonies confirmed the purity of rhizobial culture.

Composition of Lactose agar medium

Ingredients	g/l
Lactose	10.0
K_2HPO_4	0.5
NaCl	0.1
$MgSO_4.7H_2O$	0.2
Yeast Extract	1.0
Agar	20.0
Distilled water	1000 ml
pH	7.0

Composition of Benedict's reagent

Ingredients	g/l
Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	17.3
$CuSO_4.5H_2O$	17.3
Na_2CO_3 (anhydrous)	10.0
Distilled water	1000 ml

3.1.4 Isolation of *Trichoderma harzianum* cultures and their multiplication.

Trichoderma harzianum was isolated from the rhizospheric soil of shaftal plants. For this, 10gm of soil sample was taken in 90 ml sterilized water blanks and dilutions (10^{-1},10^{-2},10^{-3},10^{-4}) were made. One ml of suspension from each dilution was poured in to sterilized Petriplates. Potato Dextrose agar (PDA) with rose bengal (15-20ml) was added to the plates and rotated clockwise and anticlockwise. These plates were incubated at 30°C for 3 days. This procedure has been repeated 2-3 times. Then green coloured radiating colonies appeared on the plate. Then these cultures were purified on PDA medium by taking isolated fungal green colony. The culture of *T. viride* was procured from Department of Plant Pathology, PAU, Ludhiana. These cultures were maintained on PDA slants and stored in refrigerator. These isolates were subcultured after every 15 days throughout the period of investigation.

Composition of PDA medium

Ingredients	g/l
Potato	250
Dextrose	20
Agar	20
Distilled water	1000 ml
pH	5.0

Rose Bengal was also added in to PDA medium during isolation. The medium was sterilized by autoclaving at 15 psi pressure (121°C) for 20 minutes.

3.2 SCREENING OF *RHIZOBIUM LEGUMINOSARUM* BV *TRIFOLII* AND *TRICHODERMA HARZIANUM*

Ten isolates of *Rhizobium leguminosarum* bv *trifolii*, 4 isolates of *T. harzianum* and an isolate of *T. viride* were tested for the efficiency to fix nitrogen as well as to study their effect on growth characters of Shaftal (*Trifolium resupinatum* L.) variety Sh – 69.

Earthern pots were washed with water and then cleaned with alcohol. Soil was sterilized and filled in pots (3 Kg/pot). Healthy and viable seeds of Shaftal variety Sh-69 were surface sterilized with 0.1 per cent $HgCl_2$ for two minutes followed by dipping in ethanol for 2-3 seconds. These seeds were thoroughly washed with distilled water to remove the sterilizing agents. Surface sterilized seeds (5.0g) were inoculated with 3 days old broth (1x 10^8 cells/ml approx.) of 10 different isolates of *Rhizobium.*

Carrier based cultures of *T. harzianum* namely TH-1, TH-2, TH-3 and TH-4 and *T. viride* were prepared by using sterilized charcoal powder. Surface sterilized seeds were inoculated with *T. harzianum* and *T. viride* @ 1.0g/5.0g seeds. Each treatment had 5 replications. Uninoculated plants served as control.

Various observations were taken i.e. number of nodules /plant, dry weight of nodules /plant, fresh and dry weight of shoot and root, total chlorophyll contents, nitrate reductase activity and nitrogen content of plant.

3.3 STANDARDIZATION OF GROWTH CONDITIONS FOR *TRICHODERMA HARZIANUM* ISOLATES.

a. Effect of pH: 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5,7.0 and 7.5.

Czapeck dox broth (CDB) of following composition was used for the experiment.

Composition of Czapeck Dox Agar Medium

Ingredients	g/l
Sodium nitrate ($NaNO_3$)	2.0
Dipotassium hydrogen orthophosphate (K_2HPO_4)	1.0
$MgSO_4$	0.5
KCl	0.5
$FeSO_4$	0.01
Sucrose	30.0
Distilled water	1000 ml
Agar	20.0
pH	5.5

Czapeck dox broth (100ml) was taken in 250ml flasks and pH was adjusted to 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5. The flasks were inoculated with bits (5mm) of *Trichoderma harzianum* (TH-1). Four replications of each treatment were kept. Inoculated flasks were incubated at 28±1°C under stationary conditions for growth. Mycelial dry weight (mg) was recorded after filtration.

b. Effect of temperature: 5, 10, 15, 20, 25, 30, 35 and 40°C

Czapeck dox broth (100ml) was taken in 250ml flasks and inoculated with bits (5mm) of *Trichoderma harzianum* (TH-1). Four replications of each treatment were kept. Inoculated flasks (pH 5.5) were incubated at 5, 10, 15, 20, 25, 30, 35 and 40°C under stationary conditions for growth. Mycelial dry weight (mg) was recorded after filtration. Similarly, colony size of *T. harzianum* isolate (TH-1) was also measured using PDA. Approx. 5mm disc of *T. harzianum* was placed in the centre of Petriplate containing PDA. These plates were incubated at different temperatures viz. 5, 10, 15, 20, 25, 30, 35 and 40°C for time periods i.e. 24, 48, 72, 96,120, 144, 168 and 192 hours. The colony size was measured (cm).

c. Effect of different Carbon and nitrogen sources

Glucose, fructose, sucrose and maltose as carbon and Ammonium nitrate, ammonium sulphate, potassium nitrate and urea as a source of nitrogen were used to study the growth of *T. harzianum* (TH-1) and *T. viride*. Czapeck dox broth (CDB) was supplemented with given carbon and nitrogen sources (pH 5.5)

and inoculated with approx. 5mm discs of *T. harzianum* (TH-1) and *T. viride.* Inoculated flasks were incubated at 28±1°C under stationary conditions. Four replications of each treatment were kept and mycelial dry weight (mg) was recorded after filtration.

3.4 ANTAGONISTIC EFFECT OF CULTURE AND CULTURE FILTRATE OF *TRICHODERMA HARZIANUM* ISOLATES ON PATHOGENIC FUNGI (*SCLEROTINIA SCLEROTIUM*)

As no disease appeared on foliage and in roots in any plot of Shaftal (*Trifolium resupinatum* L.) variety Sh-69. So no experiment on antagonistic activity was carried out.

3.5 MEASUREMENT OF CHITINASE ACTIVITY OF DIFFERENT ISOLATES OF *TRICHODERMA HARZIANUM* AND *T. VIRIDE*

For this experiment, two *Trichoderma* spp viz. *T. harzianum* isolates TH-1, TH- 2, TH-3, TH-4 and *Trichoderma viride* TV were used for measuring chitinase activity. All the isolates were grown for 7 days at 28±1°C on a Czapeck dox medium (modified) containing:

Ingredients	g/l
KNO_3	10.0
KH_2PO_4	5.0
$MgSO_4\,7H_2O$	2.5
$FeCl_3$	2.0mg
Crab shell chitin (sigma)	10.0
Distilled water	1000ml
pH	6.0

The culture medium containing the enzyme of interest was separated from biomass by filtration and centrifugation at 8000 rpm (4°C) for 10min. The proteins were concentrated by vortex evaporator (Buchler instruments). The culture filtrate was assayed for enzyme activity using protocol as mentioned below with five replications. The protein estimation was done following the method of Lowry *et al* (1951). A brief procedure is given below for protein estimation.

3.4.1 Estimation of total proteins (Lowry *et al* 1951)

Reagents used were

a. 2 per cent Na_2CO_3 in 0.1N NaOH

b. 0.5 per cent $CuSO_4.5H_2O$ in 1 per cent sodium citrate

c. Mixed a+b in the ratio of 50:1 respectively.

d. Folin and phenol reagent adjusted to 1N (diluted to equal volume of water)

e. Bovine Serum Albumin (BSA) as reference standard.

Procedure

Known aliquot of protein extract was diluted to 1ml with distilled water and 5ml of reagent (c) was added. Mix the contents on vortex mixture. After 10 min 0.5ml of folin phenol reagent was added. The contents were mixed again. After 30 min, the absorbance of the developed color was assayed at 520nm on Bausch and lamb Spectronic 20 colorimeter using BSA (20-100µg) as reference standard (Fig. 1).

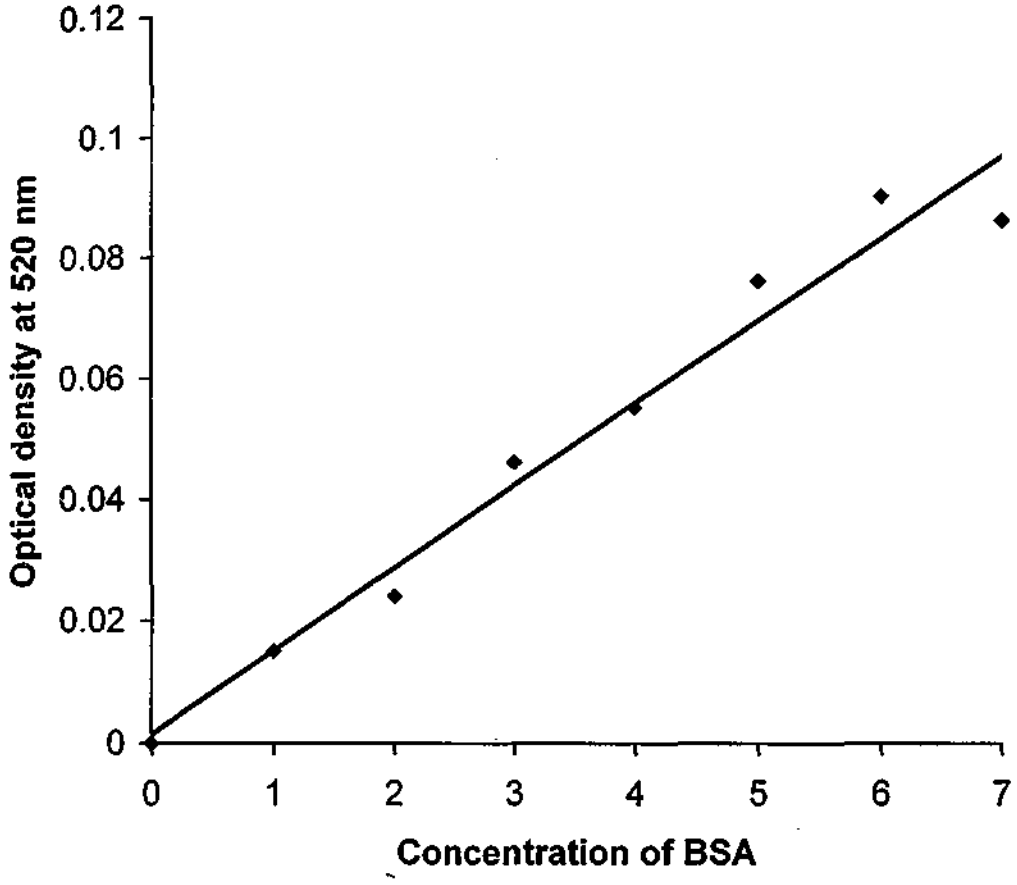


Fig. 1 Standard curve for total proteins

Chitinase

Colloidal chitin was prepared from raw chitin based on method of Shimakara and Takiguchi (1988). Enzymatic hydrolysis of colloidal chitin was assayed following the release of free N-acetylglucosamine (NAG) from colloidal chitin (Ohtakara 1988). The reaction mixture containing 1ml of 0.5 per centcolloidal chitin, 2ml of McIlvaine's buffer (equal volume of 0.2M disodium hydrogen phosphate and 0.1M citric acid, pH 4.0) and 1ml of culture filtrate was incubated for 20 min at 37°C in a shaker bath and reaction was stopped by boiling for 3min. After centrifugation of this mixture (2000 rpm for 30 min) 1.5ml of supernatant was mixed with 2ml of potassium ferricyanide reagent (0.05% potassium ferricyanide in 0.5M sodium carbonate)was heated in boiling waterbath for 15 min. The amount of NAG released was estimated from absorbance of reaction mixture at 420 nm standard (Fig. 2).

One unit of enzyme activity (CU) is defined as release of 1μmol acetylglucosamine/ml of culture filtrate/minute.

Characterization : Electrophoretic separation

Polyacrlamide disc-gel electrophoresis was used for separation and detection of enzyme (Davis 1964).

Reagents used

a. 0.25 Tris and 1.92M glycine buffer stock solution (pH 8.3): Dissolved 6.0g of hydroxymethylaminomethane (Tris) and 28.8g glycine in distilled water. Mixed and diluted the same with distilled water to 500ml. For use as tank

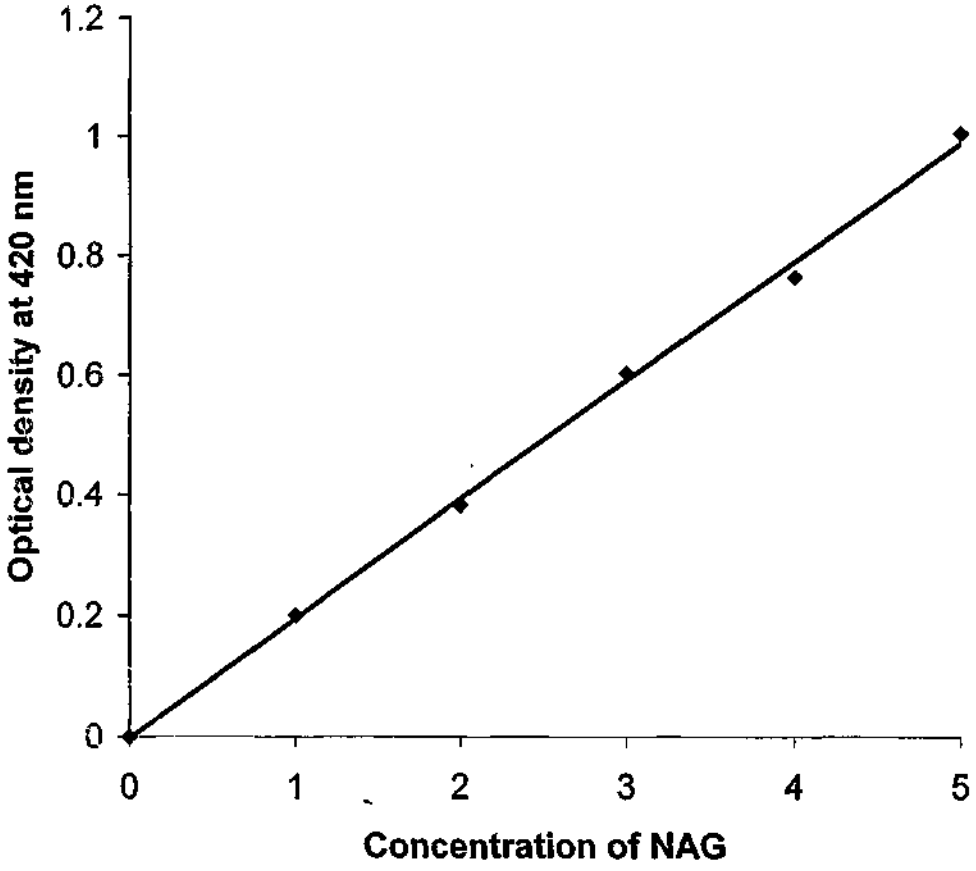


Fig. 2 Standard curve for NAG

buffer the stock solution was diluted 10 times before electrophoresis.

b. 0.375 M Tris – HCl buffer (pH 8.9): Dissolved 36.6g of Tris in 48 ml 1N HCl and 0.24ml. TEMED and diluted it upto volume of 100ml with distilled water. This buffer is filtered through Whatmann No.1 filter paper and stored at 4°C.

c. Acrylamide solution: 30g was dissolved in approximately 50ml distilled water and 0.8g bis-acrylamide in approximately 10ml distilled water.The two solutions were mixed and volume made in 100ml.

d. Ammonium persulphate solution: A solution of 0.28 per cent ammonium persulphate in distilled water was prepared. Freshly prepared solution was used

e. Fixative solution: A 12.5 per cent solution of trichloroacetic acid (TCA) in distilled water was used as fixative.

f. Protein Staining Solution Commassie blue (0.05%) was dissolved in 7.0 per cent aqueous acetic acid and 10 per cent methanol. The solution was filtered and used for staining of protein bands.

g. Destaining Solution: A solution containing methanol, acetic acid and distilled water in the ratio of 50:10:40 respectively was used.

Preparation of running gel

The running gel contains (7.5% acrylamide) was prepared by mixing reagents a, b, c, d, and distilled water in the ratio of 1:2:4:1 (v/v). The solution was poured in to electrophoresis tube (sealed with parafilm at one end). Gel in tubes

was allowed to polymerize at room temperature for 30 min.

Preparation of samples

0.5 ml Cell free extract was added to 0.5ml (w/v) 40 per cent glycerol solution in 0.02M Tris $-H_2SO_4$ buffer (pH 7.6) and 50 ul 0.05 per cent (w/v) bromophenol blue for preparation of samples for loading on the gels.

Running of gels

The gel tubes were fixed in gel electrophoresis and pre -run with Tris-glycine buffer using a current flow of 2mA/tube for 3 hours at 4°C to remove unpolymerised acrylamide and TEMED. Then the gels were loaded with sample (100µl) and 200V till the marker dye had moved the other end .The gels were gently removed and processed for protein banding pattern.

Staining of gels

The proteins were stained with coomassie blue for one hour and destained with a solution-containing methanol, acetic acid and distilled water in the ratio of 50:10: 40 respectively till the protein bands appeared.

3.6 EFFECT OF CULTURE FILTRATE OF DIFFERENT ISOLATES OF *TRICHODERMA HARZIANUM* AND *TRICHODERMA VIRIDE* ON SEED GERMINATION OF SHAFTAL (*TRIFOLIUM RESUPINATUM* L.) VARIETY SH-69

Viable and healthy seeds of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 were surface sterilized with 0.1 per cent $HgCl_2$ and thoroughly washed with distilled water to remove traces of sterilizing agent. Thirty seeds were inoculated with culture filtrate of *Trichoderma harzianum*- 1,2,3,4 and *T. viride*

and placed on sterilized Petriplates containing filterpapers. Dilutions of culture filtrates ranging from 10^{-1}-10^{-4} were used to seed treatments with four replications. Observations such as seed germination, root length, fresh weight and dry weight were recorded.

3.6.1 Effect of *Trichoderma harzianum* isolate (TH-1) on the growth of *Rhizobium leguminosarum* bv *trifolii*.

For this experiment, 250ml flasks containing 100ml of Yeast extract mannitol broth (YEM) were used. These was sterilized and inoculated with 2ml *R. leguminosarum* bv *trifolii* isolate (R-1) and culture filtrate of *Trichoderma harzianum* isolate (TH-1) in different proportions:

YEM Broth	+	*Rhizobium* (R-1)	+	*T. harzianum* (TH-1)
100ml	+	2ml	+	_
98ml	+	2ml	+	2ml
96ml	+	2ml	+	4ml
94ml	+	2ml	+	6ml
92ml	+	2ml	+	8ml
90ml	+	2ml	+	10ml

After inoculation the flasks were placed on the rotary shaker for 5 days then the O.D. was taken at 520 nm.

3.7 EFFECT OF EFFICIENT ISOLATE OF *R. LEGUMINOSARUM* BV. *TRIFOLII* AND ISOLATE OF *T. HARZIANUM* INDIVIDUALLY AND IN COMBINATION ON VARIOUS GROWTH CHARACTERISTICS AND NITROGEN FIXATION IN SHAFTAL (*TRIFOLIUM RESUPINATUM* L.) VARIETY SH-69 UNDER FIELD CONDITION.

A field experiment was conducted to examine the effect of efficient isolate of *R. leguminosarum* bv. *trifolii*, isolate of *T. harzianum* and *T. viride* individually and in combination on various growth characteristics and nitrogen fixation in Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under field condition. Efficient isolate of *Rhizobium leguminosarum* bv *trifolii* (R-1), isolate of *T. harzianum* (TH-1) and one isolate of *T. viride i.e.* TV (carrier based) were used for the experiment. In all, eight treatments were kept with four replications each. Plot size was 22 sq. m. Various observations were taken i.e. number of nodules/plant, fresh and dry weight of nodules/plant, chlorophyll contents of leaves, leghaemoglobin content of nodules, nitrate reductase and nitrogen content of shoot during the growth period.

3.7.1 Fresh and dry weight of shoot and root

Plants were uprooted and roots were separated from shoots and fresh weights were recorded. The shoots and roots were dried in an oven at 60°C for getting their dry weights.

3.7.2 Number of nodules and their dry weight

The plants were carefully removed from soil with their root system intact. Then these were washed with running water. For dry weights of nodules,

nodules were detached and dried at 60°C for two days.

3.7.3 Leghaemoglobin content

The Leghaemoglobin content of nodules was estimated by the method given by Wilson and Reisenauer (1963).

a. Nodule Extraction

Fresh nodules (0.5g) were crushed in 10ml round bottom centrifugation tube containing 3ml of Drabkin solution. The suspension was then centrifuged at 1000 x g for 15 minutes to settle down the particles of nodule tissue. This supernatant was transferred to 10ml volumetric flask. Then nodule tissue was extracted thrice more. This supernatant was added to the first flask. The total volume was made to 10ml with Drabkin solution. Then it was mixed and again centrifuged at 20000 x g for 30 minutes.

b. Determination of Leghaemoglobin content

The optical density of clear supernatant was read on a spectronic – 20 at 540nm against Drabkin solution.

3.6.4 Standard curve for Leghaemoglobin content

Drabkin solution was used for the dilution of standard cyanmethaemoglobin (CMHb) to give 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg of haemoglobin (Hb) per 10 ml. This solution was read with spectronic-20 at 540nm against drabkin solution (Fig. 3)

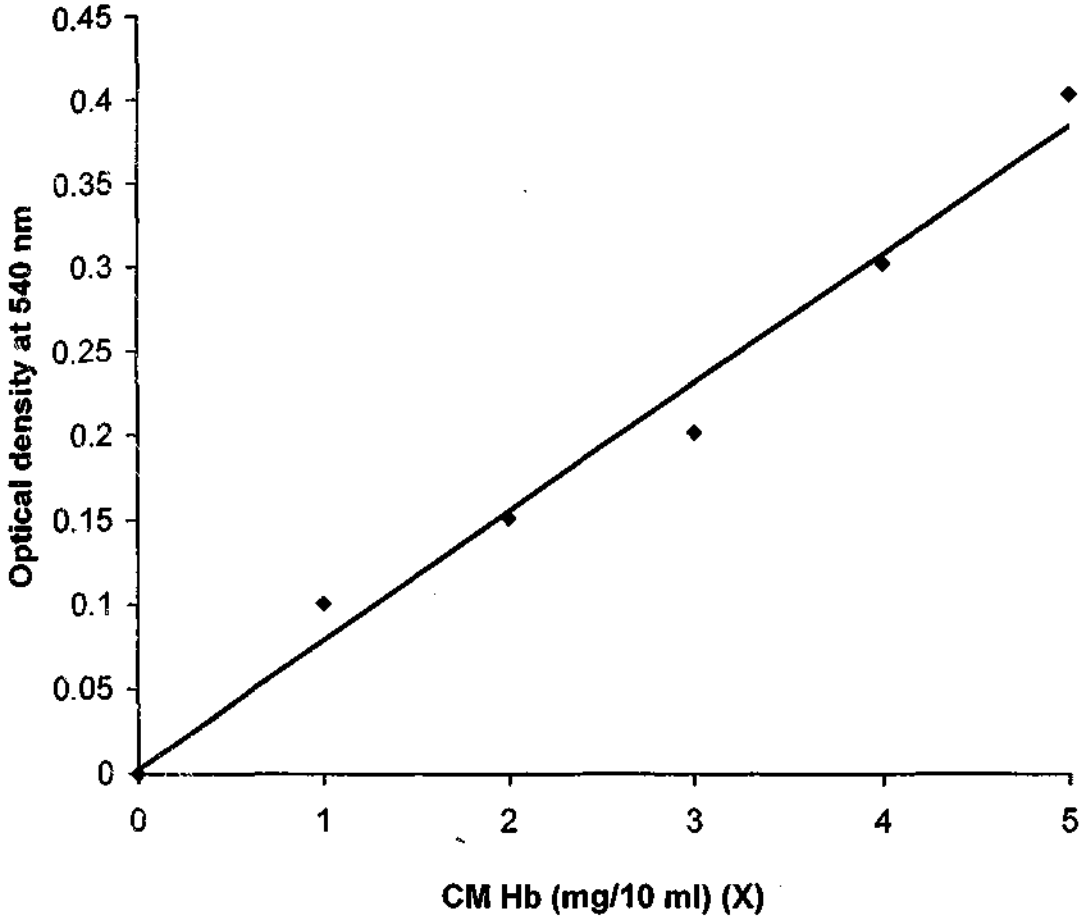


Fig. 3 Standard curve for Cyanmet haemoglobin

Composition of Drabkin solution

Ingredients	mg
Potassium cyanide (KCN)	52mg
Potassium ferricyanide [$K_3Fe(CN)_6$]	108mg
Sodium bicarbonate ($NaHCO_3$)	1mg
Distilled water	1000ml

3.7.4 Chlorophyll Estimation

Chlorophyll contents of leaves were estimated by the method of Witham *et al* (1971).

a. Chlorophyll extraction

Fully expanded leaves (0.5g) were washed and placed in a clean mortar. Then 20ml of acetone was added to it and tissue was grounded to fine pulp and Magnesium carbonate ($MgCO_3$) was added to facilitate the grinding. This solution was centrifuged at 4000 rpm for 5 minutes. After that supernatant was separated and mixed with freshly made 15ml of 80 per cent acetone. The solution was again centrifuged and added to the flask containing the first extract and the final volume of flask was made to 50ml with 80 per cent acetone.

b. Chlorophyll estimation

Spectronic-20 (Baush and Lomb) was used for measuring optical density of chlorophyll extract at 645nm and 663nm using 80 per cent acetone as solvent blank. The chlorophyll contents was calculated on the basis of mg of

chlorophyll/g of leaf tissue extracted by using following equations:

mg Chlorophyll 'a'/g tissue = 12.7 (D_{663}) - 2.69 (D_{645}) x V/1000 x w

mg Chlorophyll 'b'/g tissue = 22.9 (D_{645}) - 4.68 (D_{663}) x V/1000 x w

mg Chlorophyll 'total'/g tissue = 20.2 (D_{645}) + 8.02 (D_{663}) x V/1000 x w

D : Optical density of chlorophyll extract at the specific indicated wavelength.

V : Final volumes of the 80 per cent Acetone chlorophyll extract solution.

w : Fresh weight in grams of tissue extracted

3.7.5 Nitrate reductase activity (Jaworski 1971)

Uppermost fully expanded leaves (200g) were rinsed with water and suspended in a screw cap vial (25ml) containing 5ml of medium of the following composition:

0.1M Phosphate buffer (pH - 7.5)

0.02M KNO_3

5 per cent Propanol

Two drops of chloroamphenicol (0.5mg/ml).

These vials were sealed and incubated in the dark for one hour at 25°C, which resulted in the release of nitrite in to the medium. This was determined by treating 0.5ml of aliquot with 0.3ml each of 1 per cent sulphanilamide in 3 M HCl and 0.02 per cent NEDH (1ml N-naphthyl-ethylene-diamine hydrochloride). After about 20 minutes, the solutions were diluted with 4ml of water and O.D. was measured on spectronic -20 at 540nm. A blank without leaves served as control.

3.7.6 Standard curve for Nitrate reductase

A standard solution of KNO_2 was diluted to get different concentrations i.e. 0, 8, 16, 24, 32 and 40μM NO_2/ml. The colour was developed by adding one ml of 1 per cent sulphanilamide and NEDH solution to 2ml of the sample. The intensity of colour was measured by spectronic-20 at 540nm (Fig. 4).

3.7.7 Nitrogen Estimation (Burris and Wilson 1957)

The plants were dried in an oven at 60°C for about 2 hours. These were ground in to fine powder and stored for nitrogen estimation. One-gram powder was taken and placed in Kjeldahl flask (250ml). Then 5.0g of digestion mixture containing 1.0g of $CuSO_4$ and 2.0g of K_2SO_4 were added to digestion flask along with 5ml of Mercuric sulphate solution. (12ml of concentrated H_2SO_4 diluted to 100ml and was added to 10g of mercuric oxide). Concentrated H_2SO_4 (15ml) and few beads were added and flask was heated until solution becomes clear. At this stage whole of the nitrogen was converted to $(NH_4)_2SO_4$). The flask was cooled and 25ml water was added. These contents were transferred to 250 ml volumetric flask and the volume was made to 100ml. The boric acid was back titrated with the standard acid (N/100 H_2SO_4) using 2 drops of indicator (0.25g of methylene blue, 0.375g of methyl red and 300ml of 95% ethanol). This turned from green through grey to purple grey colour as the end point.

$$\text{per cent N in the sample} = (T-B)\ N.\ \frac{1.4}{S}$$

T : Sample titration

N : Normality of standard

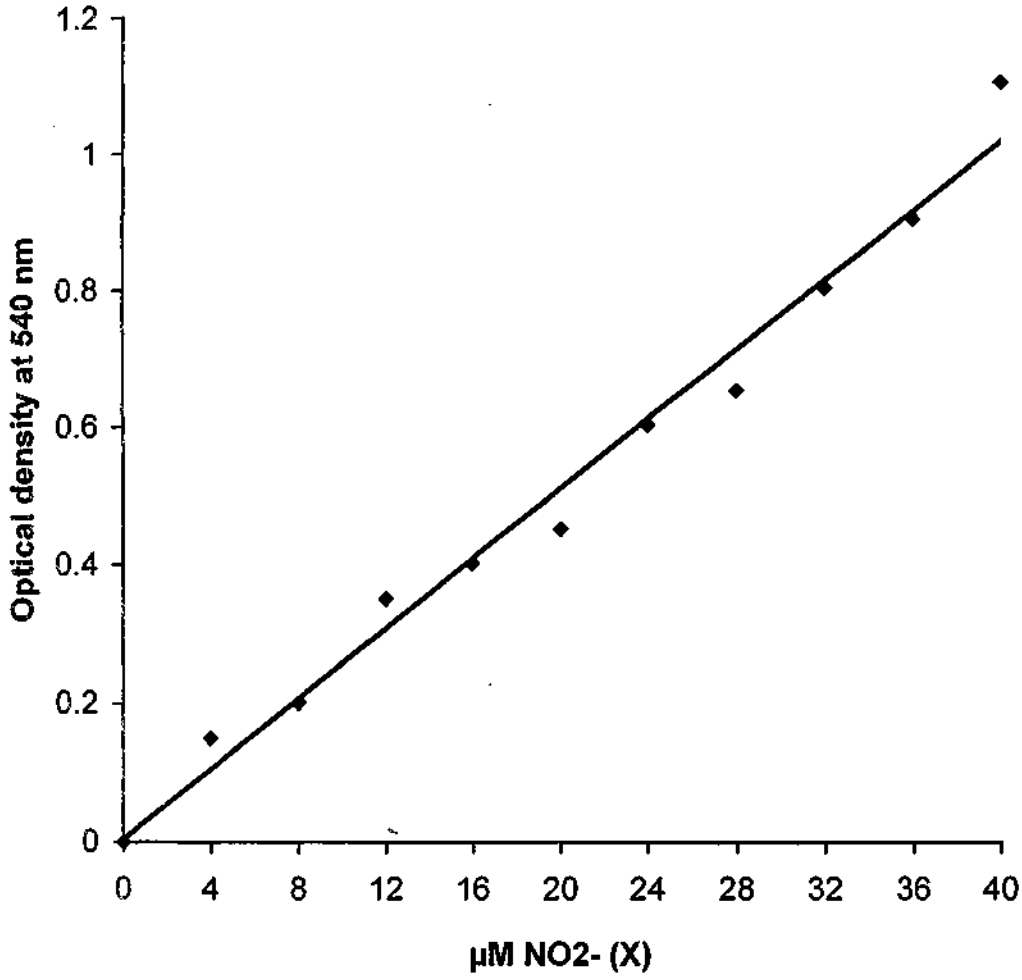


Fig. 4 Standard curve for nitrate reductase

B : Blank Titration

S : Sample weight

3.8 TO STUDY COMPETITIVENESS OF *R. LEGUMINOSARUM* BV *TRIFOLII* AND ESTABLISHMENT OF *T. HARZIANUM* IN RHIZOSPHERE OF SHAFTAL (*TRIFOLIUM RESUPINATUM* L.) VARIETY SH-69

The antibiotic markers of the organisms were recognized by standard technique. The Petriplates containing Yeast extract mannitol medium were inoculated with a suspension of cell. The cell suspension was spread uniformly over the medium with a glass spreader. Antibiotic discs were placed aseptically on the seeded medium and the plates were incubated at 28±1°C for 3 days. The size of inhibition zone was recorded. Total 8 antibiotics were used i.e. (Erythromycin 15 mcg, Tetracycline 30mcg, Kanamycin 10mcg, Streptomycin 10 mcg, Gentamycin 10mcg, Vancomycin 30 mcg Nalidixic acid 30 mcg and Chloramphenicol 30mcg). The size of inhibition zone was measured.

Nodules from inoculated and control plants were taken, washed, surface sterilized and then crushed. Each nodule sap was pour plated on YEMA plates with and without selective antibiotics. Per cent Competitiveness was calculated from:

Per cent Competitiveness = per cent marked nodules in inoculated - per cent marked nodules in control.

For studying establishment of *Trichoderma,* rhizospheric soil was serially diluted and plated on PDA. After incubation, CFU /gm of rhizospheric soil

was recorded.

3.9 SCREENING OF SUBSTRATES FOR MULTIPLICATION OF *T. HARZIANUM* AND *T. VIRIDE*

Different substrates i.e. wheat straw, wheat bran, rice straw, bagasse, potato peels, boiled potato, compost, fruit peel (orange), fruit juice extract (orange) and tea leaves waste. All the substrates were weighed, soaked in water and placed for one day so that the total moisture content should be 60-70 per cent. All these substrates were added in wide mouthed tubes and sterilized at 121°C (15 psi) pressures. These tubes were inoculated with a bit of *Trichoderma harzianum* and *T. viride* (approx. 5mm) separately and incubated at 28±1°C. Four replications for each treatment were kept and CFU/g was taken after ten days of incubation.

Chapter – IV

RESULTS AND DISCUSSION

4.1 *RHIZOBIUM* AND *TRICHODERMA* ISOLATES

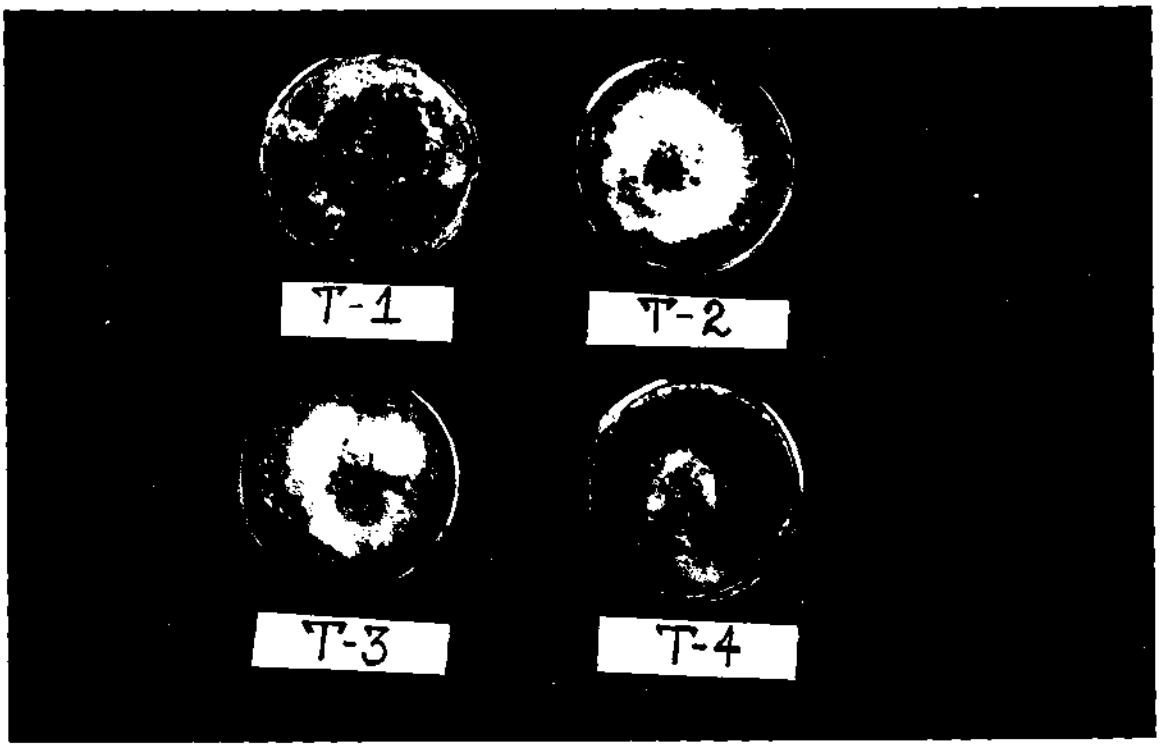
Rhizobium leguminosarum bv. *trifolii* and *Trichoderma harzianum* isolates used in the present investigation were isolated from the nodules and rhizosphere of Shaftal (*Trifolium resupinatum* L.) respectively. In total 10 isolates of *R. leguminosarum* bv. *trifolii* and 4 isolates of *T. harzianum* were purified and characterized. Rhizobia are rod shaped, Gram-negative, Ketolactose negative and non spore former. *Trichoderma harzianum* isolated from rhizospheric soil was identified on the basis of colony characters. Culture was at first white and later on turned dark green as shown in (Plates 1 and 2). The mycelium of *T. harzianum* was septate and colonies were hyaline, bearing repeatedly bent flask shaped phialides. The conidia were globbose or subglobbose or short obovoid. When sporulating freely, they had branched conidiophores, the branches almost at right angles to the main axis.

4.2 SCREENING OF EFFICIENT ISOLATES OF *R.LEGUMINOSARUM* BV. *TRIFOLII* AND *T. HARZIANUM* ON SHAFTAL (*TRIFOLIUM RESUPINATUM* L.) VARIETY SH-69 UNDER POT CULTURE CONDITIONS

A pot culture experiment using sterilized soil was conducted to screen efficient isolates of *R. leguminosarum* bv. *trifolii* and *T. harzianum* and *T. viride* on Shaftal (*Trifolium resupinatum* L.) variety Sh-69. Ten isolates of *R. leguminosarum* bv. *trifolii* were examined for their effect on nitrogen fixation and

Plate 1 : Culture of *Trichoderma harzianum*

Plate 2 : Four isolates of *Trichoderma harzianum*


T-1
T-2
T-3
T-4

growth character of Shaftal. Surface sterilized seeds (5.0g) were inoculated with 3 days old broth (1×10^8 cells/ml approx.) of 10 different isolates of *R. leguminosarum* bv. *trifolii*. The cultures (carried based) of *T. harzianum* namely TH-1, TH-2, TH-3, TH-4 and TV were prepared. Surface sterilized seeds were inoculated with *T. harzianum* cultures @ 1.0g/5.0g seeds. Each treatment had 5 replications. Inoculated seeds were sown in earthern pots. Uninoculated plants served as control. Three cuttings were taken during the entire growth period of crop. The following observations were recorded: -

4.2.1 First cutting

First cutting was taken after 55 days of sowing.

It is obvious from the results (Table 1) that plants inoculated with *Rhizobium* isolates R-1 to R-10 significantly produced more fresh weight of shoot and root. The maximum fresh weight of shoot and root was produced by *Rhizobium* isolate R-1 (7.44 g and 1.23 g) respectively. The Dry weight of shoot and root was also observed to be maximum in R-1 isolate i.e. 2.16 g and 0.352 respectively followed by isolate R-6 and R-5. The combination of R-1 + TH-1 significantly produced more fresh and dry weights of shoot and root (i.e. 6.92 g; 1.27g and 1.22 g; 0.353 g) respectively as compared to *Trichoderma* alone.

The results (Table 1) shows that number of nodules also varies considerably with the *Rhizobium* isolates. No nodulation was found in the treatments containing *T. harzianum* and *T. viride* as the pots contained steam sterilized soil. Maximum number of nodules/plant and their dry weight was

Table 1: Effect of *R. leguminosarum* bv *trifolii* and *Trichoderma harzianum* (alone and in combination) on various growth characteristics of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under pot culture conditions using sterilized soil. Data represent means of 5 replications

Treatments	Fresh wt of shoot/plant(g)	Fresh wt of root/plant(g)	Dry wt of shoot/plant(g)	Dry wt of root/plant(g)	Number of Nodules	Dry wt of Nodules(g)
Control	4.23	1.11	1.13	0.326	-	-
R-1	7.44	1.23	2.16	0.352	41	0.026
R-2	6.33	1.13	1.29	0.339	33	0.023
R-3	5.32	1.14	1.39	0.329	29	0.021
R-4	6.21	1.16	1.21	0.331	32	0.023
R-5	7.13	1.22	1.33	0.350	39	0.034
R-6	7.40	1.23	1.31	0.352	38	0.024
R-7	5.35	1.16	1.16	0.329	27	0.020
R-8	4.93	1.19	1.38	0.347	26	0.022
R-9	5.41	1.17	1.12	0.336	31	0.024
R-10	6.11	1.18	1.18	0.339	26	0.023
TH-1	6.21	1.12	1.21	0.330	-	-
TH-2	6.30	1.13	1.19	0.333	-	-
TH-3	6.41	1.12	1.21	0.333	-	-
TH-4	6.31	1.13	1.21	0.331	-	-
TV	5.91	1.16	1.17	0.336	-	-
TH-1+R-1	6.92	1.27	1.22	0.353	42	0.030
TH-2+R-1	6.13	1.29	1.16	0.361	40	0.026
TH-3+R-1	6.96	1.27	1.20	0.362	40	0.025
TH-4+R-1	6.84	1.26	1.23	0.373	39	0.024
TV+R-1	6.10	1.24	1.21	0.363	37	0.021
TH-1+TH-2	6.13	1.30	1.25	0.372	-	-
TH-1,2,3,4,+R-1	7.49	1.31	1.26	0.371	41	0.025
TH-1,2,3,4+TV+R-1	7.49	1.31	1.29	0.382	41	0.026
C.D at 5%	1.016	0.322	0.322	0.425	1.63	0.506

R: *Rhizobium;* TH : *Trichoderma harzianum;* TV : *Trichoderma viride*

obtained in case of *Rhizobium* isolate R-1. Isolate R-1 significantly produce more number of nodules (41 nodules/plant) and their dry weight 0.026 g/plant. There was maximum Leghaemoglobin content in the nodules formed by R-1 isolate (1.34 mg/0.5 g of nodules). In combination of *Rhizobium* with all the isolates of *Trichoderma* there was non-significant difference in the leghaemoglobin content. There was no nodulation in control and *Trichoderma* treatments. The Leghaemoglobin content of R-1 to R-10 was in the range of 1.14 mg to 1.34 mg/0.5g of nodules.

Rhizobium inoculation significantly increased chlorophyll and nitrogen content of shoot as compared to control (Table 2). Maximum chlorophyll content was obtained in the *Rhizobium* isolate R-1 (1.43 mg). Nitrogen content of shoot was maximum in R-1 (1.51%) followed by R-7 (1.49%) and R-6 (1.40%). The combination of *Rhizobium* and *T. harzianum* produced nitrogen (1.46%).

There were no significant differences in the nitrate reductase activity of leaves among different nodules. The maximum NRA of leaves (0.59 μM NO_2/0.5 g of leaf sample) was measured in the plants inoculated with R-1 and R-6 isolate followed by R-8 (0.52 μM).

4.2.2 Second cutting

Second cutting was taken after 80 days of sowing.

Results indicate (Table 3) that the fresh weight of shoot and root was found to be maximum in case of *Rhizobium* isolate R-1(6.99g/plant) followed by isolate R-5 (6.93g/plant). The fresh and dry weights of shoot and root were more

Table 2: Effect of *Rhizobium* and *T. harzianum* (alone and in combination) on various growth characters of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under pot culture conditions using sterilized soil (after 55 days of sowing). Data represent mean of 5 replications.

Treatments	Leghaemoglobin content of nodules (mg/0.5g of nodules)	Total chlorophyll contents of leaves (mg/0.5g of fresh leaves)	Nitrate reductase activity of leaves μM/0.5g of leaf sample.	Nitrogen content of shoot (%)
Control	-	0.92	0.37	-
R-1	1.34	1.43	0.59	1.51
R-2	1.23	1.31	0.48	1.43
R-3	1.23	1.33	0.46	1.36
R-4	1.19	1.29	0.39	1.31
R-5	1.21	1.40	0.50	1.38
R-6	1.23	1.39	0.49	1.40
R-7	1.14	1.36	0.47	1.49
R-8	1.22	1.36	0.52	1.46
R-9	1.21	1.29	0.51	1.32
R-10	1.18	1.31	0.47	1.16
TH-1	-	1.40	0.39	-
TH-2	-	1.39	0.36	-
TH-3	-	1.37	0.39	-
TH-4	-	1.31	0.38	-
TV	-	1.28	0.38	-
TH-1+R-1	1.33	1.49	0.59	1.42
TH-2+R-1	1.31	1.43	0.61	1.33
TH-3+R-1	1.29	1.49	0.61	1.40
TH-4+R-1	1.27	1.47	0.59	1.39
TV+R-1	1.20	1.26	0.57	1.38
TH-1+TH-2	-	1.52	0.62	-
TH1, 2,3,4+R-1	1.29	1.50	0.61	1.46
TH-1, 2,3,4 +TV+R-1	1.28	1.51	0.64	1.46
CD at 5% level	0.361	0.600	0.196	0.205

R :*Rhizobium*
TH:*Trichoderma harzianum*
TV:*Trichoderma viride*

Table 3: *Effect of R. leguminosarum* bv *trifolii* and *Trichoderma harzianum* (alone and in combination) on various growth characteristics of Shaftal (*Trifolium resupinatum* L.) variety sh-69 under pot culture conditions using sterilized soil (after 80 days of sowing). Data represent means of 5 replications.

Treatments	Fresh wt of shoot/plant(g)	Fresh wt of root/plant(g)	Dry wt of shoot/plant(g)	Dry wt of root/plant(g)	Number of Nodules	Dry wt of Nodules(g)
Control	4.19	1.12	1.29	0.320	-	-
R-1	6.99	1.26	2.09	0.396	40	0.026
R-2	6.11	1.19	2.01	0.341	32	0.024
R-3	5.12	1.20	1.56	0.322	30	0.020
R-4	5.33	1.14	1.54	0.361	33	0.024
R-5	6.93	1.31	1.16	0.370	32	0.026
R-6	6.01	1.24	1.29	0.361	37	0.028
R-7	5.62	1.19	1.13	0.328	32	0.020
R-8	5.11	1.20	1.09	0.336	30	0.019
R-9	5.10	1.21	1.24	0.392	36	0.019
R-10	5.12	1.21	1.29	0.329	34	0.030
TH-1	6.16	1.13	1.26	0.340	-	-
TH-2	6.13	1.19	1.38	0.360	-	-
TH-3	6.80	1.16	1.29	0.392	-	-
TH-4	6.10	1.20	1.31	0.322	-	-
TV	5.24	1.19	1.16	0.360	-	-
TH-1+R-1	6.82	1.43	1.34	0.391	39	0.031
TH-2+R-1	5.96	1.32	1.19	0.343	36	0.031
TH-3+R-1	6.20	1.16	1.30	0.371	38	0.030
TH-4+R-1	6.11	1.39	1.29	0.383	38	0.029
TV+R-1	6.20	1.34	1.24	0.381	34	0.029
TH-1+TH-2	6.85	1.29	1.23	0.362	-	-
TH-1,2,3,4,+R-1	6.96	1.28	1.31	0.373	39	0.020
TH-1,2,3,4+TV+R-1	7.10	1.27	1.30	0.399	38	0.029
C.D at 5%	1.16	0.328	0.249	0.211	NS	NS

R: *Rhizobium;* TH : *Trichoderma harzianum;* TV : *Trichoderma viride*

in case of *T. harzianum* isolates as compare to control. Among combination treatment (TH-1, 2, 3 and 4 + TV+R-1) gave maximum fresh weights of shoot and root (7.10g and 1.27 g) respectively. The dry weights of shoot and root was also found to be maximum in case of *Rhizobium* isolate R-1 i.e. 2.09 g/plant and 0.396 g/plant respectively followed by combination of TH-1+R-1 significantly produced more fresh and dry weights of shoot and root i.e. (6.82 g; 1.43g; and 1.34 g and 0.391 g respectively) as compared to *Trichoderma* alone. Among *Trichoderma* treatments TH-1 produced more fresh and dry weight of shoot and root i.e. (6.16g; 1.13g and 1.26; 0.340 g respectively).

Number of nodules were maximum in case of R-1 (40/plant). Nodulation was not observed in treatments of *T. harzianum* and *T. viride* and control due to sterilization. Leghaemoglobin content of nodules was found to be maximum in case of R-2 (1.09 mg/0.5g of nodules). There was no significant increase in the leghaemoglobin content of nodules in the combination of *Rhizobium* and *Trichoderma* spp (Table 4).

The maximum chlorophyll contents of leaves were observed in plants inoculated with *Rhizobium* isolate R-1 (1.36 mg). There was non-significant increase in the chlorophyll contents of leaves in case of *T. harzianum* and *T. viride* treatments as compared to control. Among *Trichoderma* treatments, TH-1 isolate had shown more chlorophyll contents i.e. 1.35 mg/0.5g of fresh leaves. There was non-significant increase in chlorophyll contents in the treatments containing *Trichoderma* and *Rhizobium* isolates. The treatment TH-1,2,3,4 + TV+R-1 gave

Table 4 : Effect of *Rhizobium* and *T. harzianum* (alone and in combination) on various growth characters of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under pot culture conditions using sterilized soil (after 80 days of sowing). Data represent mean of 5 replications.

Treatments	Leghaemoglobin content of nodules (mg/0.5g of nodules)	Total chlorophyll contents of leaves (mg/0.5g of fresh leaves)	Nitrate reductase activity of leaves M/0.5g of leaf sample.	Nitrogen content of shoot (%)
Control	-	0.81	0.42	-
R-1	1.01	1.36	0.44	1.53
R-2	1.09	1.24	0.41	1.51
R-3	1.06	1.21	0.41	1.52
R-4	1.03	1.23	0.36	1.49
R-5	1.07	1.30	0.39	1.42
R-6	1.08	1.31	0.46	1.48
R-7	1.03	1.26	0.41	1.39
R-8	1.04	1.32	0.41	1.36
R-9	1.01	1.19	0.38	1.46
R-10	1.06	1.19	0.42	1.42
TH-1	-	1.35	0.43	-
TH-2	-	1.13	0.41	-
TH-3	-	1.22	0.42	-
TH-4	-	1.11	0.49	-
TV	-	1.14	0.44	-
TH-1+R-1	1.06	1.19	0.42	1.42
TH-2+R-1	1.08	1.28	0.41	1.37
TH-3+R-1	1.01	1.26	0.41	1.38
TH-4+R-1	1.10	1.29	0.42	1.51
TV+R-1	1.07	1.32	0.32	1.32
TH-1+TH-2	-	1.31	0.42	-
TH-1, 2,3,4,+R-1	1.04	1.29	0.45	1.49
TH-1, 2,3,4+TV+R-1	1.07	1.36	0.41	1.49
CD at 5% level	0.193	0.499	0.164	0.163

R :*Rhizobium*
TH:*Trichoderma harzianum*
TV:*Trichoderma viride*

chlorophyll content (1.36 mg).

There was non-significant difference in the nitrate reductase activity of leaves. Maximum nitrate reductase activity was obtained in case of R-1 and in combination of *Trichoderma* and *Rhizobium* TH-1+R-1 (0.42μM).

Nitrogen content was maximum in case of *Rhizobium* isolate R-1 (1.53%). In the combination of *Trichoderma* and *Rhizobium,* nitrogen content was non-significantly lesser than *Rhizobium* alone but more than *Trichoderma* alone treatments.

4.2.3 Third cutting

Third cutting was taken after 110 days of sowing.

Maximum fresh weight of shoot and root was obtained in case of R-1 (6.99g/plant and 1.26g/plant respectively). Maximum dry weight of shoot and root was obtained in case of *Rhizobium* isolate (R-1) was 2.16g/plant and 0.369g respectively (Table 5). The combination of *Trichoderma* and *Rhizobium* (TH-1+ R-1 and TH-1, 2,3,4 + R-1) treatments significantly produced more fresh and dry weight of shoot and root as compared to other *Trichoderma* alone treatments.

Isolate R-1 significantly produce more number of nodules and more dry weight i.e. (31 nodules/plant and 0.029 g) respectively. Nodulation do not occur in case of *Trichoderma* treated plants as the pots contained steam sterilized soil.

Maximum chlorophyll contents have been observed in isolate (R-2 1.36 mg/0.5 g of leaves). *Trichoderma harzianum* inoculation showed more

Table 5: **Effect of *Rhizobium* and *T. harzianum* (alone and in combination) on various growth characters of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under pot culture conditions using sterilized soil (after 110 days of sowing). Data represent mean of 5 replications.**

Treatments	Fresh wt of shoot/plant (g)	Fresh wt of root/plant (g)	Dry wt of shoot/plant (g)	Dry wt of root/plant (g)	Number of Nodules	Dry wt of Nodules (mg)
Control	3.92	1.92	1.22	0.316	-	-
R-1	6.99	1.26	2.16	0.369	31	0.029
R-2	6.03	1.03	2.00	0.336	21	0.019
R-3	5.01	0.99	1.91	0.316	21	0.018
R-4	5.16	1.03	1.92	0.359	23	0.020
R-5	6.21	1.25	1.99	0.361	29	0.021
R-6	6.14	1.13	2.13	0.315	29	0.020
R-7	4.93	1.10	1.51	0.299	30	0.030
R-8	5.21	1.10	1.59	0.296	21	0.018
R-9	4.69	1.12	1.43	0.316	23	0.019
R-10	5.03	1.14	1.49	0.296	29	0.019
TH-1	6.32	1.16	2.33	0.313	-	-
TH-2	6.21	1.14	2.21	0.326	-	-
TH-3	6.11	1.14	2.19	0.314	-	-
TH-4	6.23	1.16	2.11	0.331	-	-
TV	4.91	1.19	1.50	0.316	-	-
TH-1+R-1	6.23	1.21	2.13	0.394	33	0.018
TH-2+R-1	5.24	1.23	1.91	0.331	23	0.020
TH-3+R-1	4.91	1.12	1.60	0.319	33	0.032
TH-4+R-1	4.91	1.21	1.53	0.299	33	0.031
TV+R-1	5.13	1.19	1.54	0.314	23	0.019
TH-1+TH-2	6.11	1.26	2.16	0.310	-	-
TH-1,2,3,4,+R-1	6.73	1.32	2.23	0.317	36	0.031
TH-1, 2,3,4 +TV+R	6.21	1.31	2.26	0.329	34	0.030
C.D at 5% level	0.271	0.600	0.388	NS	1.64	0.573

R : *Rhizobium,* TH : *Trichoderma harzianum,,* TV : *Trichoderma viride*

chlorophyll content (Table 6) as compared to control isolate TH-3 (1.39 mg).

Nitrate reductase activity showed significantly less difference in all the treatments. *Rhizobium* isolate (R-1) showed maximum (0.44 µM/0.5 g of leaf sample) NRA activity. *Trichoderma* treatments do not show significantly high value of NRA activity. The results obtained by the combination of *Rhizobium* and *Trichoderma* gave almost similar values that ranges from (0.31- 0.49 µM). The treatment TH-1, 2, 3, 4 + R-1, gave 0.49 µM/0.5 g of leaf sample and TH-1, 2, 3, 4, + TV + R-1, gave 0.43 µM) which was non-significant increase in NRA activity.

Nitrogen content was maximum in *Rhizobium* (R-1) isolate (1.47%). The nitrogen content of *Rhizobium* isolate ranges from 1.36 to 1.50 per cent. The pots inoculated with TH-1, TH-2, TH-3 and TH-4 showed significantly lesser nitrogen content as compare to other treatments. The combination of TH-1 +R-1 and TH-1,2,3,4+R-1 gave (1.48% and 1.49%) respectively.

Three cuttings have been taken after 55 days, 80 days and 110 days respectively (Table 7). Among *Rhizobium* treatment maximum total green fodder yield was obtained in R-1 (571.0g/pot) followed by R-2 (564.0g/pot). In case of *T. harzianum* alone maximum green fodder yield was obtained in TH-1 (564.0g/pot) followed by TH-4 (547.0g/pot). The combination of TH-1, 2,3 and 4 TV + R-1 gave the green fodder yield (573.0g/pot) and TH-1+R-1 resulted in (559.0g/pot). Similarly, there was variation in plant height (Table 8). Maximum plant height in (cm) was shown by combination TH-1,2,3,4 + TV + R-1 (55.9 cm) followed by

Table 6: Effect of *R. leguminosarum* bv *trifolii* and *Trichoderma harzianum* (alone and in combination) on various growth characteristics of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under pot culture conditions using sterilized soil (after 110 days of sowing). Data represent mean of 5 replications.

Treatments	Leghaemoglobin content of nodules (mg/0.5g of nodules)	Total chlorophyll contents of leaves (mg/0.5g of fresh leaves)	Nitrate reductase activity of leaves uM/0.5gof leaf sample.	Nitrogen content of shoot (%)
Control	-	0.87	0.29	-
R-1	1.06	1.39	0.44	1.47
R-2	1.00	1.30	0.42	1.42
R-3	1.01	1.31	0.43	1.44
R-4	1.01	1.32	0.36	1.46
R-5	1.03	1.38	0.42	1.39
R-6	1.00	1.39	0.40	1.36
R-7	1.00	1.32	0.41	1.38
R-8	1.01	1.23	0.42	1.41
R-9	1.06	1.23	0.42	1.40
R-10	1.09	1.26	0.47	1.40
TH-1	-	1.37	0.32	-
TH-2	-	1.38	0.31	-
TH-3	-	1.37	0.36	-
TH-4	-	1.32	0.39	-
TV	-	1.29	0.33	-
TH-1+R-1	1.12	1.46	0.46	1.48
TH-2+R-1	1.09	1.41	0.42	1.50
TH-3+R-1	1.08	1.42	0.41	1.42
TH-4+R-1	1.12	1.46	0.42	1.36
TV+R-1	1.01	1.31	0.41	1.39
TH-1+TH-2	-	1.49	0.42	-
TH-1,2,3,4+R-1	1.10	1.51	0.49	1.49
TH-1, 2,3,4 +TV+R-1	1.07	1.49	0.43	1.48
CD at 5%	0.681	0.371	NS	0.642

R: *Rhizobium*
TH:*Trichoderma harzianum*
TV:*Trichoderma viride*

Table 7: Effect of *R. leguminosarum* bv *trifolii* and *Trichoderma harzianum* (alone and in combination) on green fodder yield of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under pot culture conditions using sterilized soil. Data represent mean of 5 replications.

Treatments	Green fodder yield (g /pot)			
	First cutting (55 days)	Second cutting (80 days)	Third cutting (110 days)	Total green fodder yield
Control	167.0	162.0	156.0	485.0
R-1	196.0	190.0	185.0	571.0
R-2	190.0	185.0	189.0	564.0
R-3	181.0	176.0	184.0	541.0
R-4	183.0	169.0	173.0	525.0
R-5	172.0	169.0	171.0	512.0
R-6	163.0	173.0	162.0	498.0
R-7	169.0	168.0	162.0	499.0
R-8	156.0	162.0	164.0	482.0
R-9	161.0	159.0	164.0	484.0
R-10	181.0	158.0	163.0	502.0
TH-1	189.0	196.0	179.0	564.0
TH-2	186.0	196.0	163.0	545.0
TH-3	179.0	191.0	173.0	543.0
TH-4	174.0	192.0	181.0	547.0
TV	163.0	195.0	184.0	542.0
TH-1+R-1	179.0	192.0	188.0	559.0
TH-2+R-1	179.0	193.0	176.0	548.0
TH-3+R-1	174.0	193.0	183.0	550.0
TH-4+R-1	181.0	182.0	189.0	552.0
TV+R-1	183.0	181.0	185.0	549.0
TH-1+TH-2	188.0	182.0	182.0	552.0
TH1,2,3,4+R-1	189.0	190.0	186.0	565.0
TH-1,2,3,4+TV+R-1	191.0	189.0	193.0	573.0
CD at 5% level				12.57

R: *Rhizobium*
TH:*Trichoderma harzianum*
TV:*Trichoderma viride*

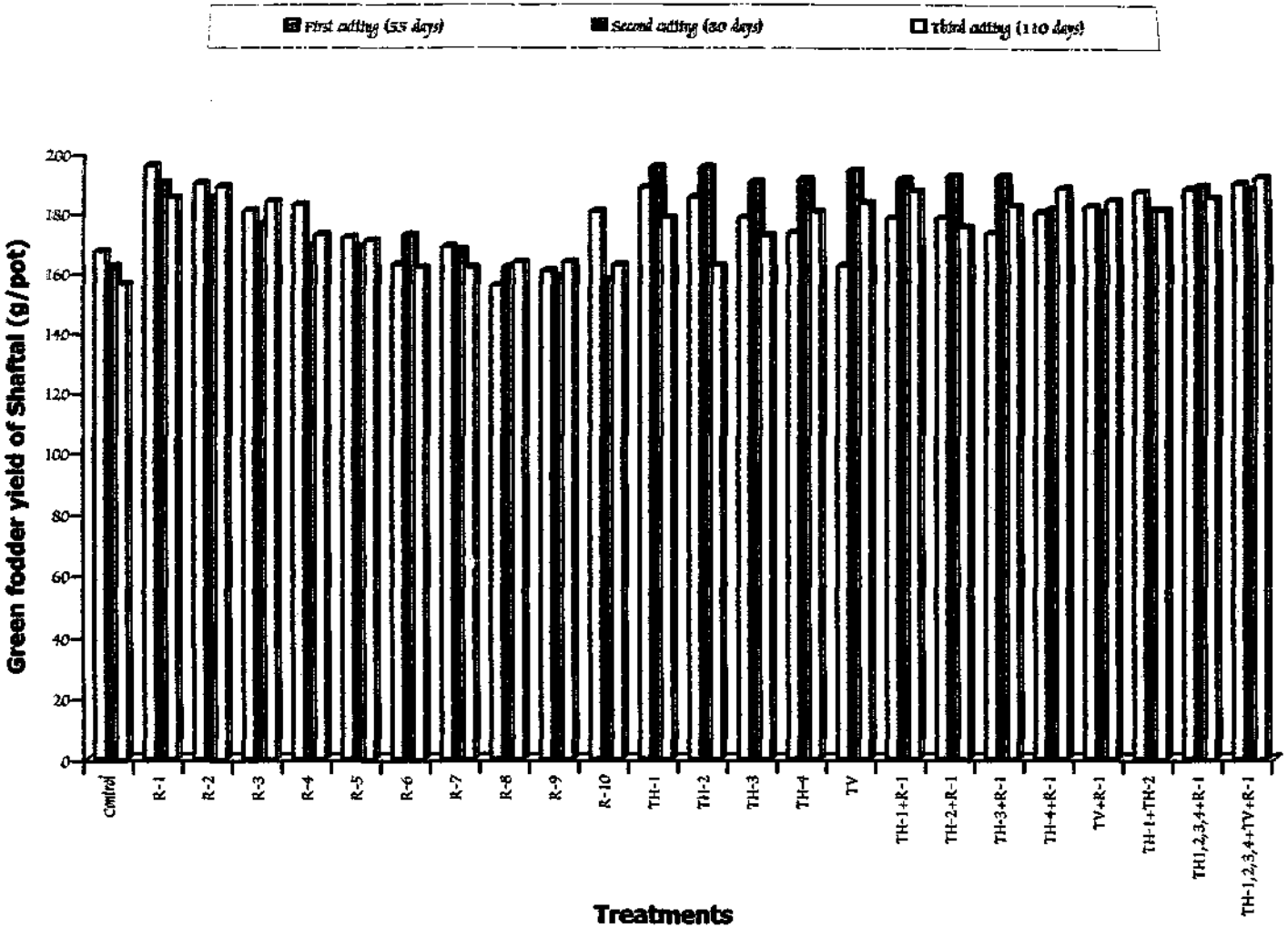


Fig. 5 Effect of *R. leguminosarum* bv *trifolii* and *Trichoderma* spp. (alone and in combination) on green fodder yield of Shaftal *(Trifolium resupinatum* L.) variety Sh-69 under pot culture conditions using sterilized soil

Table 8 : Effect of *R.leguminosarum* bv *trifolii* and *Trichoderma harzianum* (alone and in combination) plant height and green fodder yield of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under pot culture conditions using sterilized soil. Representative samples were taken for measuring plant height.

Treatments	Plant height (cm)	Total green fodder yield (g/pot)
Control	49.2	485.0
R-1	54.3	571.0
R-2	53.6	564.0
R-3	51.2	541.0
R-4	51.6	525.0
R-5	50.8	512.0
R-6	49.9	498.0
R-7	49.8	499.0
R-8	50.8	482.0
R-9	52.6	484.0
R-10	52.6	502.0
TH-1	55.8	564.0
TH-2	53.9	545.0
TH-3	52.8	543.0
TH-4	52.6	547.0
TV	53.9	542.0
TH-1+R-1	51.6	559.0
TH-2+R-1	51.2	548.0
TH-3+R-1	50.9	550.0
TH-4+R-1	50.8	552.0
TV+R-1	53.6	549.0
TH-1+TH-2	55.1	552.0
TH-1,2,3,4+R-11	54.6	565.0
TH-1, 2,3,4 +TV+R-1	55.9	573.0

R: *Rhizobium*
TH: *Trichoderma harzianum* ; TV: *Trichoderma viride*

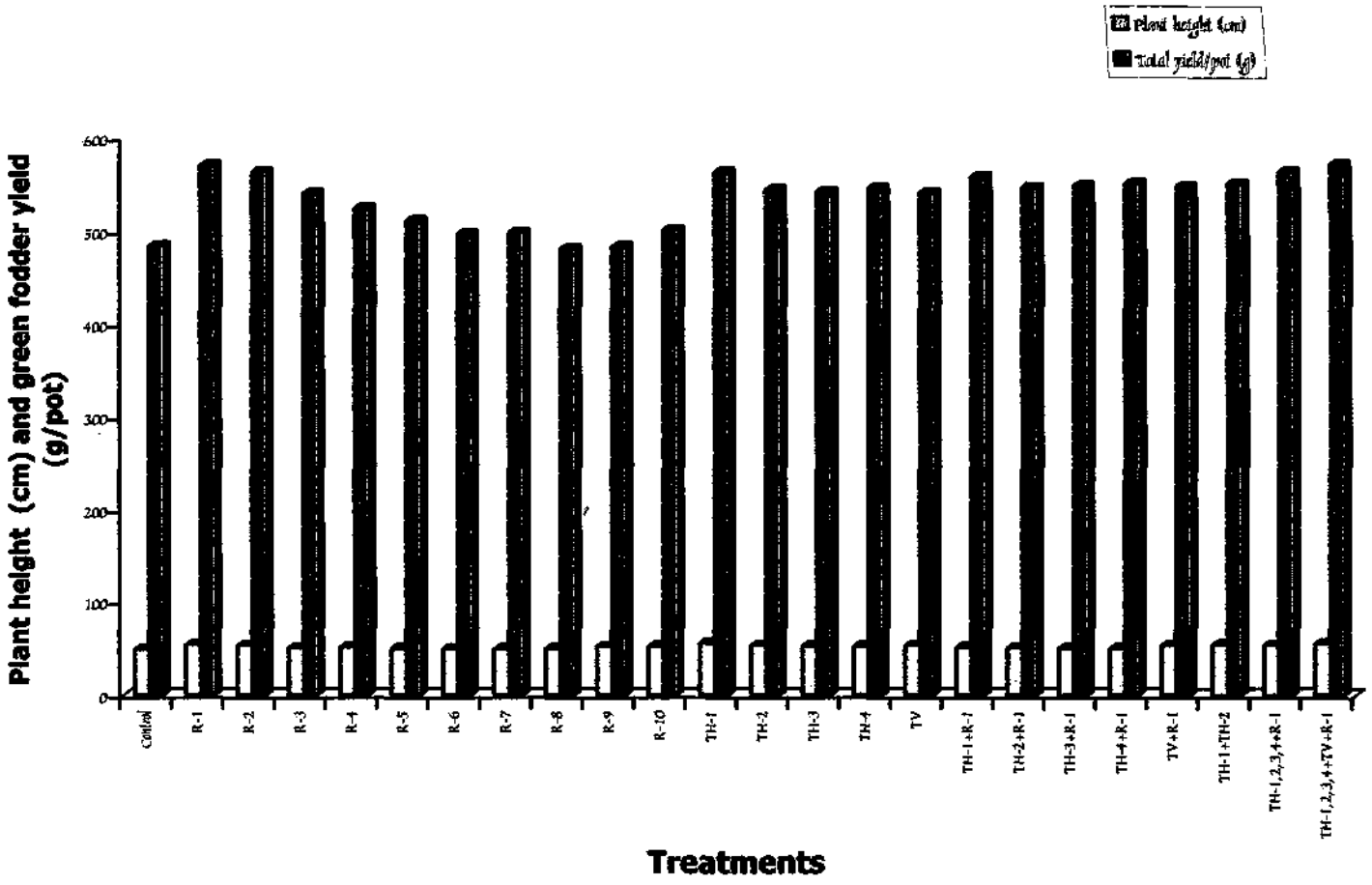


Fig. 6 Effect of *R. leguminosarum* bv *trifolii* and *Trichoderma harzianum* (alone and in combination) on plant height and green fodder yield of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 in pot culture conditions

TH-1 (55.8 cm) and R-1 (54.3 cm).

Above results obtained on strain variation among *Rhizobium* isolates was similar to the previous reports showing variation (Plate 3) in nodulation and nitrogen fixation due to inoculation with *Rhizobium* strains (Prabhakaran and Ramaswamy 1990; Brewin et al 1993; Shukla and Dixit 1996; Maldal and Ray 1999; Graham and Vance 2000 and Sindhu and Dadarwal 2000).

Inoculation with *Rhizobium* and *Trichoderma* isolates showed similar results as reported by Bhardwaj (1970). He reported the stimulatory effect of *T. lignorum* on the nodulation of *Melilotus alba* and *M. indica* in both natural and sterilized soil conditions. Kehri and Chandra (1991) observed significant increase in the weight of nodules of mungbean plants raised from *T. viride* treated seeds. Likewise, Jayaraj and Ramabadran (1999) also studied *Rhizobium* and *Trichoderma* interaction *invivo* and *invitro* in blackgram. They found non-significant increase in nodulation between *Rhizobium* alone and *Rhizobium* + *Trichoderma* treatments (Plates 4 and 5).

4.3 STANDARDIZATION OF GROWTH CONDITIONS FOR *TRICHODERMA HARZIANUM* ISOLATES.

(a) Effect of pH : [3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5]

(b) Effect of Temperature : [5, 10, 15, 20, 25, 30, 35 and 40°C]

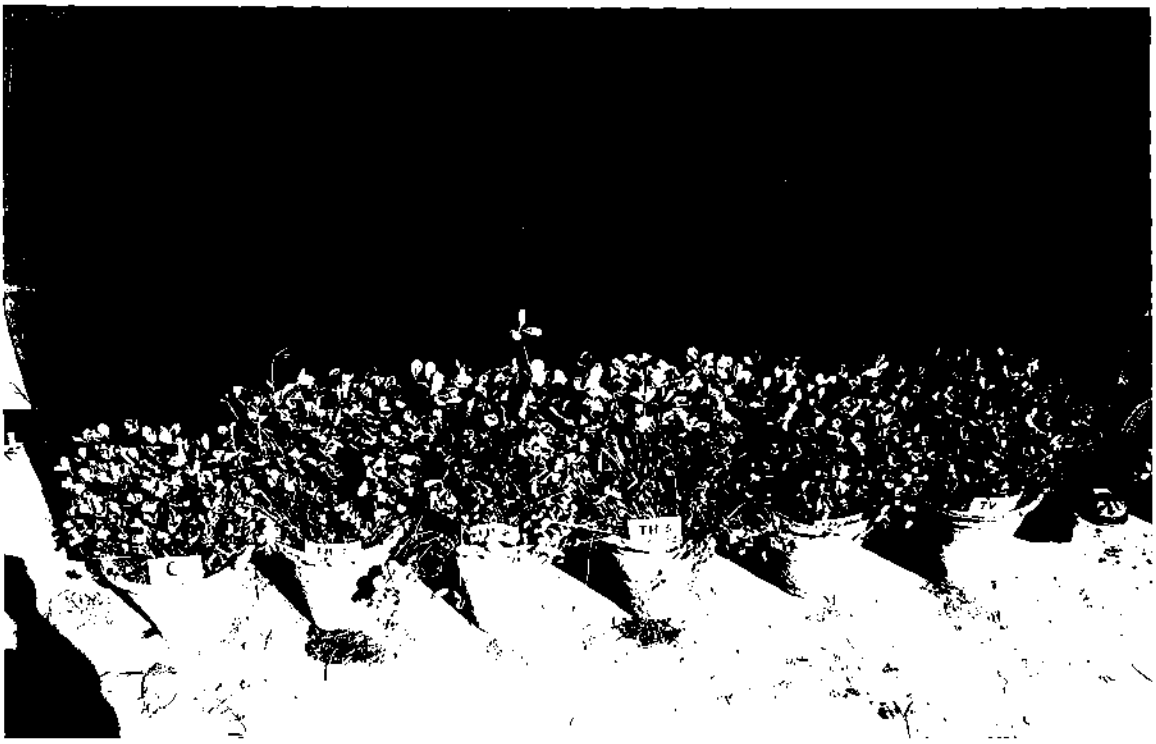
The effect of pH (pH 3.0-7.5) and temperature (5-40°C) on the growth of *Trichoderma harzianum* isolate was investigated using Czapeck Dox (CD) broth. The pH and temperature has significant effect on the growth of *T.*

Plate 3 : Effect of *Rhizobium leguminosarum* bv. *trifolii* isolates (R-1, R-5 and R-6) on various growth characteristics of Shaftal (*Trifolium resupinatum* L.) variety Sh-69


C
R-1
R-5
R-6

Plate 4 : Effect of *Trichoderma harzianum* and *T. viride* isolates on various growth characteristics of Shaftal (*Trifolium resupinatum* L.) variety Sh-69

Plate 5 : Effect of combination of *Rhizobium*, *T. harzianum* and *T. viride* isolates on various growth characteristics of Shaftal (*Trifolium resupinatum* L.) variety Sh-69



harzianum isolates. The observations were taken in terms of mycelial dry weight (mg/100 ml of CD broth). It has been observed that growth was optimum in the pH range of 5.5 to 7.5 (Table 9). Maximum growth of *Trichoderma harzianum* was observed at pH 6.5. *Trichoderma harzianum*–1 showed significantly more growth (391.1 mg) at pH 6.5 as compared to other *T. harzianum* isolates. TH–1 was followed by TH–2 which in turns followed by isolate TH-4 and then TH-3, (381.2 mg, 372.2 mg and 363.0 mg/ 100 ml of broth) respectively.

The temperature range has also showed significant effect on the growth of *Trichoderma harzianum.* The observations were taken in terms of dry weight of mycelium (mg/100 ml of broth). No growth was observed at temperature 5 and 10°C (Table 10). There is significantly optimum growth at the temperatures of 25° - 30°C. *T. harzianum* isolates TH-1 and TH-2 gave more dry mycelial weight at 30°C as compared to other two isolates i.e. (169.2 mg). *T. harzianum* isolates TH-4 and TH-3 gave the mycelial dry weight (159.0 mg and 156.0 mg) respectively at 30°C.

The effect of temperature on colony size at different time periods (24–192 hours) was also investigated. It is evident from the results obtained (Table 11) that the maximum colony size of *T. harzianum* was observed at 25°C after 192 hours (9.2 cm). The colony size was significantly more between the temperatures of 25 and 30°C as compared to other temperatures. Minimum colony size was found between 5 and 10°C at different intervals of time (Plate 6). During 24, 48, 72 hours of incubation almost negligible growth was found at temperatures range

Table 9 : Effect of different pH on growth of *Trichoderma harzianum* isolates in *in vitro* conditions at temperature (28±1°C) using Czapeck dox broth. Data represent mean of 4 replications.

pH	Mycelial dry weight in (mg)/ 100 ml broth			
	TH-1	TH-2	TH-3	TH-4
5.5	369.2	344.1	339.2	341.2
3.0	40.3	39.0	41.3	36.3
3.5	39.0	42.0	146.2	38.1
4.0	51.0	59.1	52.2	56.0
4.5	53.1	60.3	61.2	69.0
5.0	294.2	363.2	341.3	316.1
6.0	299.3	343.0	394.2	364.0
6.5	391.0	381.5	363..3	372.3
7.0	396.1	349.6	314.1	316.2
7.5	390.1	326.3	348.0	329.2
C.D at 5% level	1.69	1.66	1.61	1.63

TH: *Trichoderma harzianum*

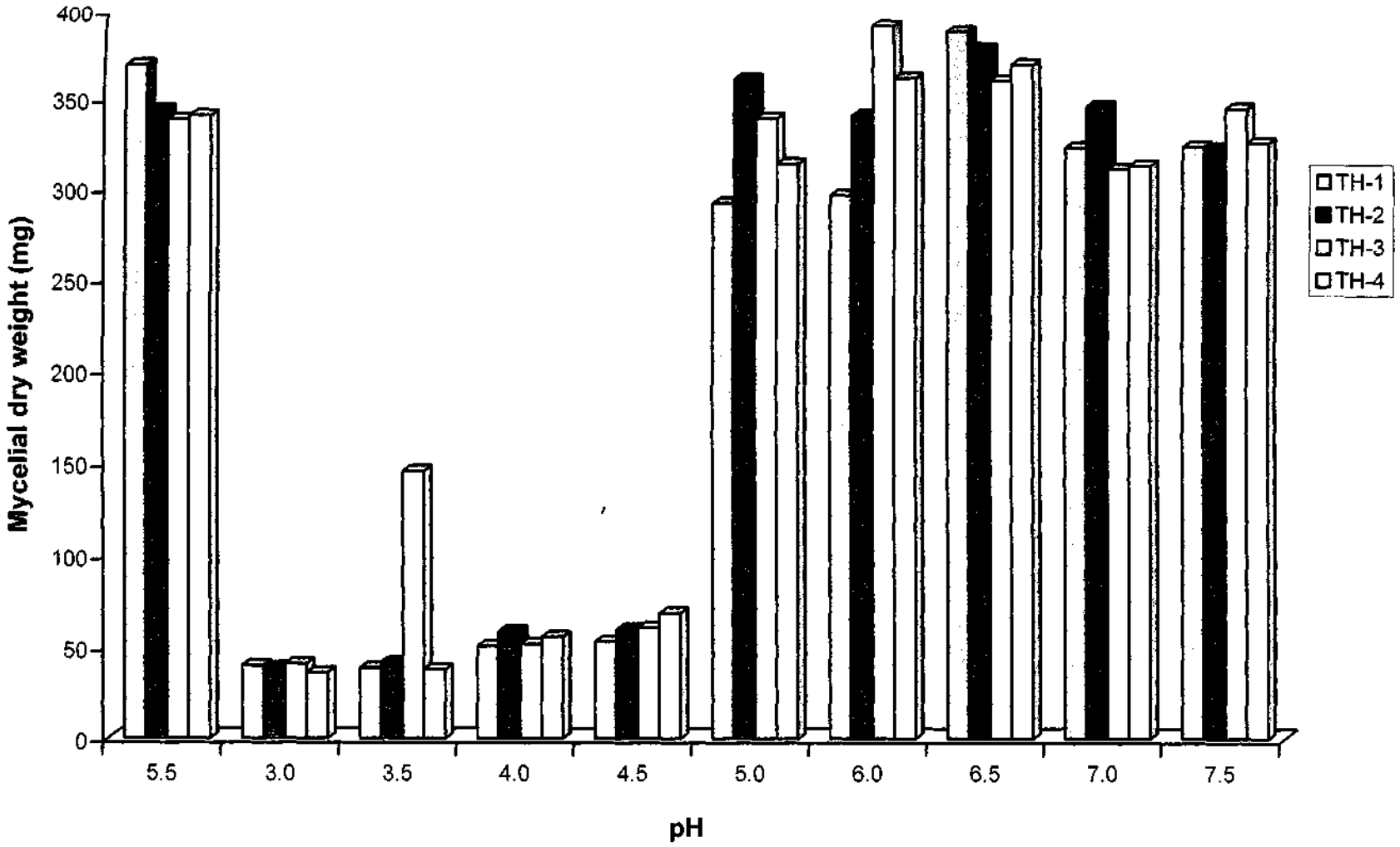


Fig. 7 Effect of different pH on growth of *Trichoderma harzianum* isolates (TH-1 - TH-4) in *in vitro* conditions using Czapeck dox broth

Table 10 : Effect of different temperatures (pH 5.5) on growth of *Trichoderma harzianum* isolate (TH-1) in *in vitro* conditions using Czapeck dox broth. Data represent mean of 4 replications.

Temperature °C	Mycelial dry weight in (mg) / 100 ml broth			
	TH-1	TH-2	TH-3	TH-4
28	165.6	159.8	164.3	164.9
5	-	-	-	-
10	-	-	-	-
15	51.2	39.2	52.2	46.3
20	143.0	129.0	131.3	140.1
25	161.0	159.1	142.0	146.0
30	169.2	169.0	156.0	159.1
35	21.1	26.3	27.1	29.5
40	21.3	25.5	25.1	29.3
CD at 5% level	1.48	1.43	1.38	1.41

TH: *Trichoderma harzianum*

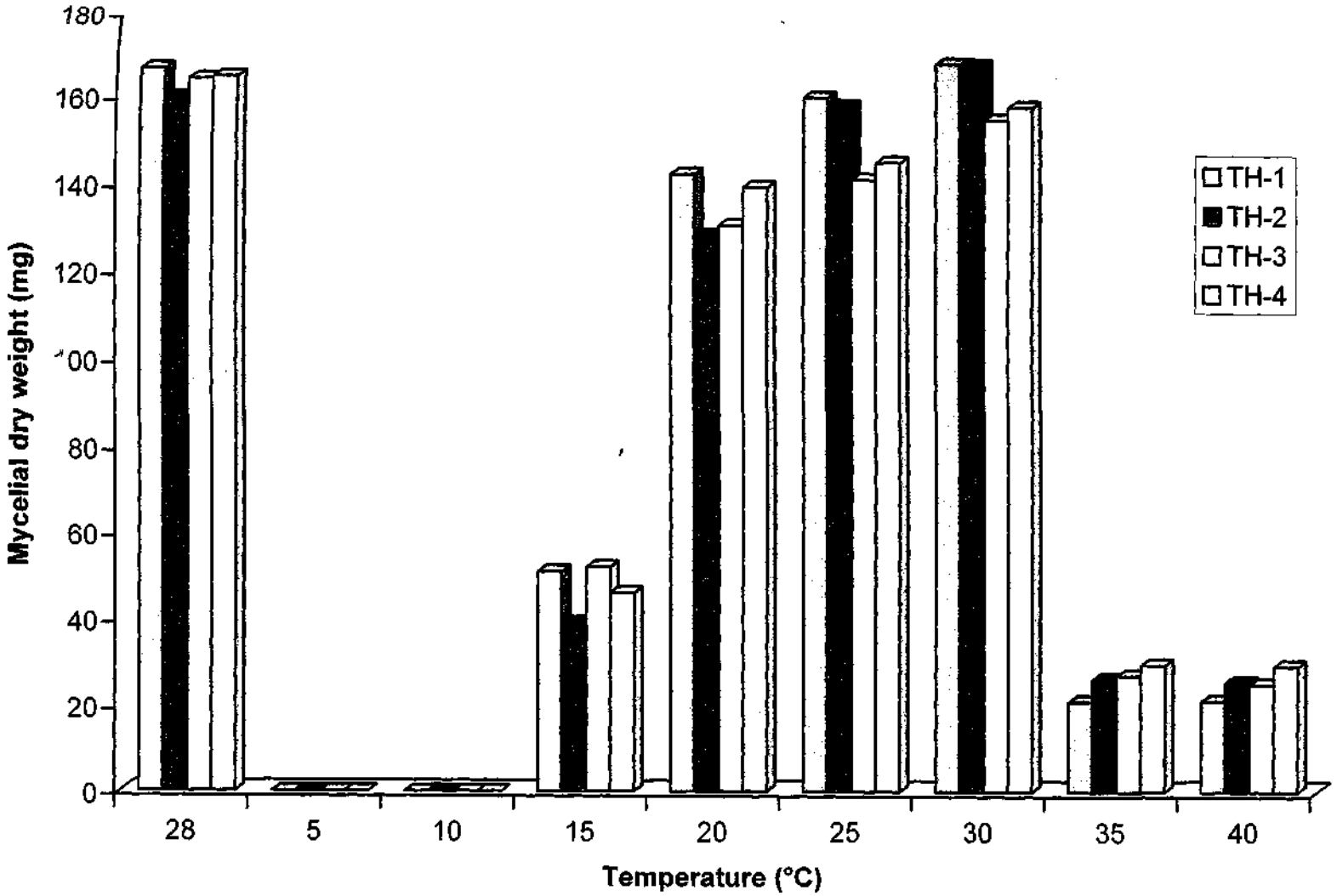


Fig. 8 Effect of different temperatures on growth of *Trichoderma harzianum* isolates (TH-1 - TH-4) in *in vitro* conditions using Czapeck dox broth

Table 11: Effect of different temperatures on the colony size (cms) of *T. harzianum* isolate (TH-1) using Potato dextrose agar. Data represent mean of 5 replications.

Temperature	Colony size (in cms) after time (in hours)							
°C	24	48	72	96	120	144	168	192
5	0	0	0.3	0.8	0.9	0.9	1.0	1.2
10	1	1	1.2	1.2	1.6	1.8	2.2	3.4
15	1	1.6	2.2	2.9	3.6	4.2	4.8	6.9
20	1.2	1.3	2.8	3.3	3.9	4.9	5.9	7.2
25	1.4	1.9	2.9	3.6	4.9	5.8	6.7	9.2
30	1.3	1.9	2.8	3.1	4.6	5.5	6.2	9.0
35	1.1	1.8	1.8	2.6	3.5	4.9	5.2	5.6
40	0	0	1.3	1.7	2.1	2.7	3.1	4.2
C.D at 5% level	0.996	0.818	0.593	0.654	0.598	0.596	0.594	0.484

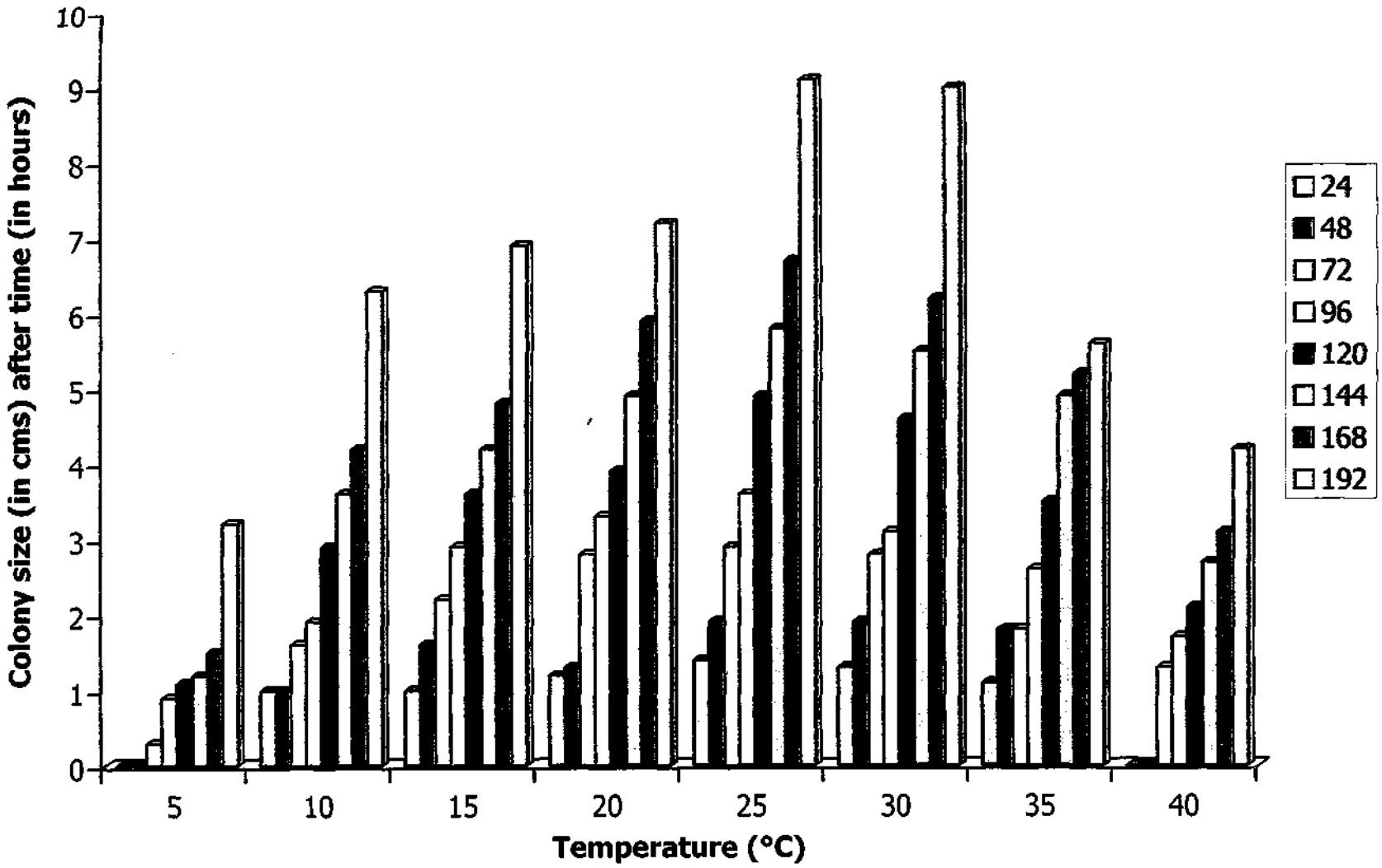
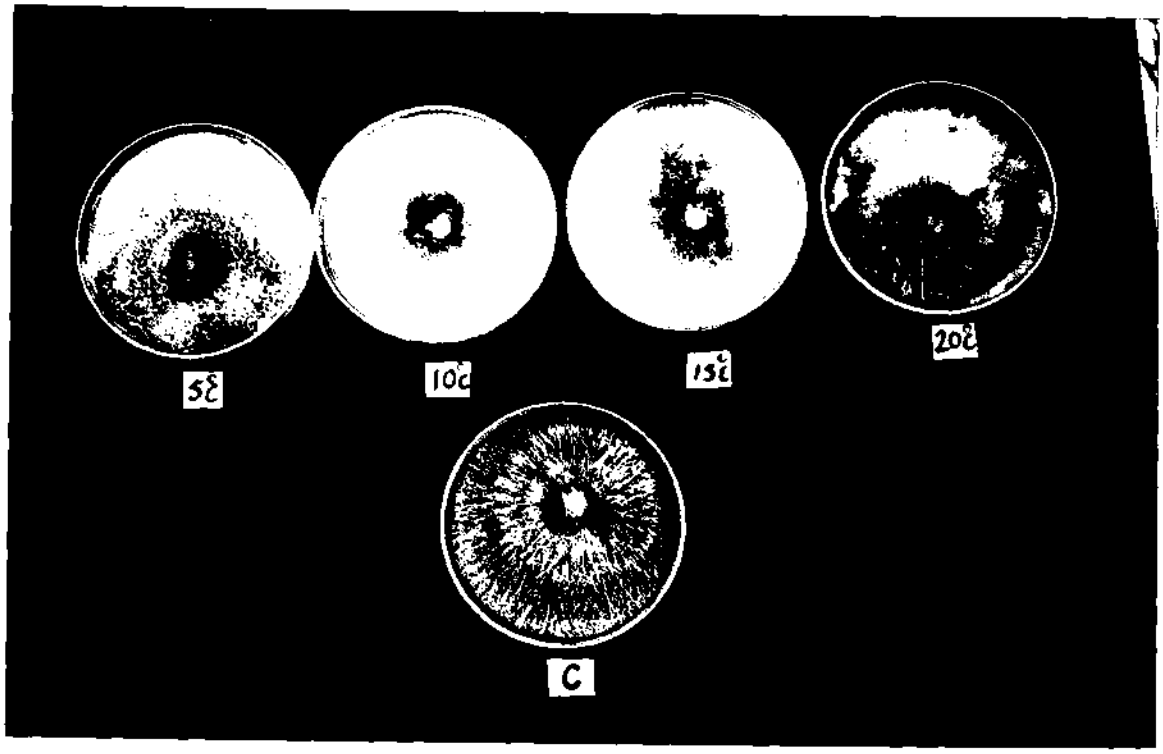
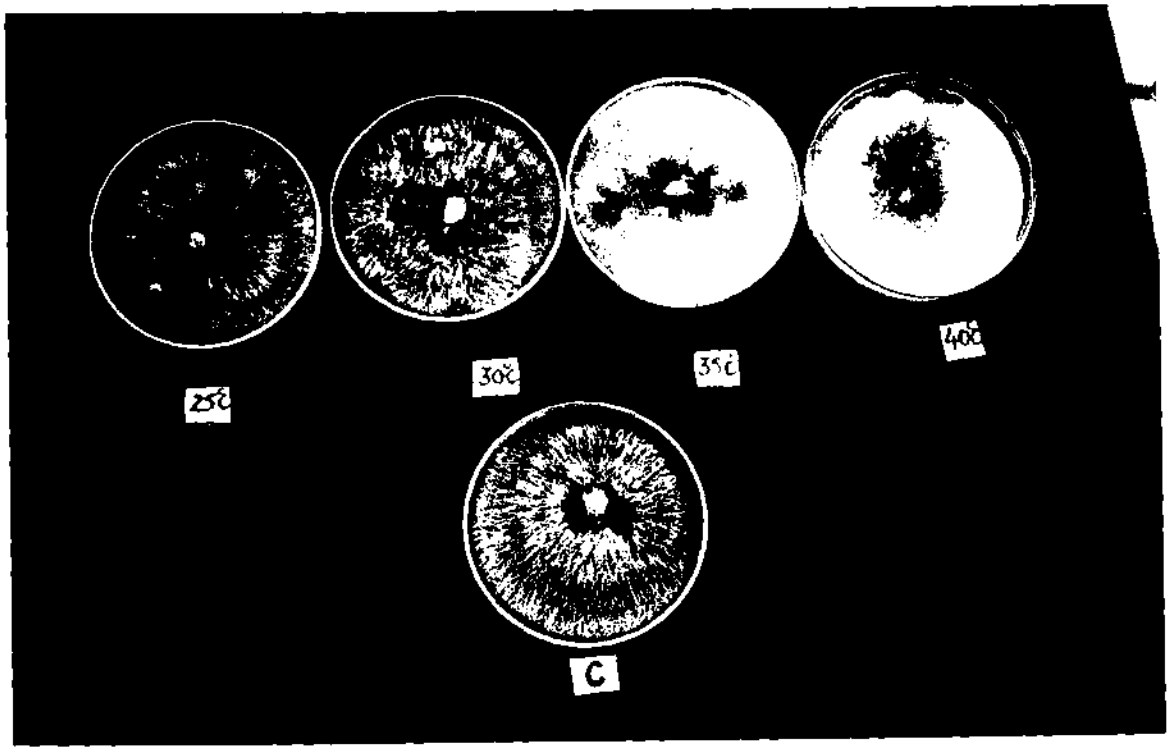


Fig. 9 Effect of different temperatures on the colony size (cms) of *T. harzianum* isolate (TH-1)

Plate 6 : Effect of different temperatures 5, 10, 15 and 20°C on colony size of *T. harzianum* (TH-1)

Plate 7 : Effect of different temperatures 25, 30, 35 and 40°C on colony size of *T. harzianum* (TH-1)


5C
10C
15C
20C
C


25C
30C
35C
40C
C

5°C and 10°C. Even at 40°C colony size was found to be very less or almost negligible up to the time period of 48 hours but then it increases and ranges between 1.3 - 4.2 cm. The temperatures of 25 – 30°C for 7-8 days was found to be significantly good as compared to other temperature (Plate 7). At 35°C growth was significantly less as compared to 30°C but was slightly more than at 40°C.

The factors like pH and temperature might affect the growth and multiplication of *T. harzianum*. In order to examine this effect, present investigation was done. The optimum growth has been obtained at the temperatures of 25-30°C at pH 6.5. Optimum range for growth was at pH 5.5 – 7.5. Likewise, Jackson *et al* (1991) conducted an experiment to study effect of pH and temperature on growth of *Trichoderma* and reported that optimum pH range was 4.5 to 6.8 and optimum temperature range was 20-30°C. Rollan *et al* (1999) reported that optimum range for temperature is 25 and 30°C. He concluded that optimum temperature also affect the biocontrol activity of *Trichoderma*. Bastos (2001) reported that optimum temperature and pH range for growth and sporulation of *Trichoderma* was 20-30°C and 5.5-7.5 respectively.

c. Effect of Carbon sources : Glucose, Fructose, Sucrose, Maltose

Another *in vitro* experiment was conducted to examine the effect of carbon on the growth of *T. harzianum* and *T. viride* isolates using Czapeck dox broth. Growth was measured by taking dry weight of mycelium (mg/100 ml of Czapeck dox broth). Data (Table 12) revealed that when glucose was used as a carbon source, growth was found to be significantly greater as compared to other

Table 12: Effect of Carbon sources on the growth of *Trichoderma harzianum* isolate (TH-1) and *T. viride* in *in vitro* conditions using Czapeck dox broth (temperature 28±1°C and incubation period 7 days). Data represent mean of 4 replications.

Carbon sources	Mycelial dry weight (mg) / 100 ml broth	
	T. harzianum	*T. viride*
CD broth without C source	-	-
Glucose + $NaNO_3$	199.2	169.3
Fructose + $NaNO_3$	192.1	153.0
Sucrose + $NaNO_3$	190.0	164.0
Maltose + $NaNO_3$	80.3	108.1
C.D at 5% level	1.818	1.716

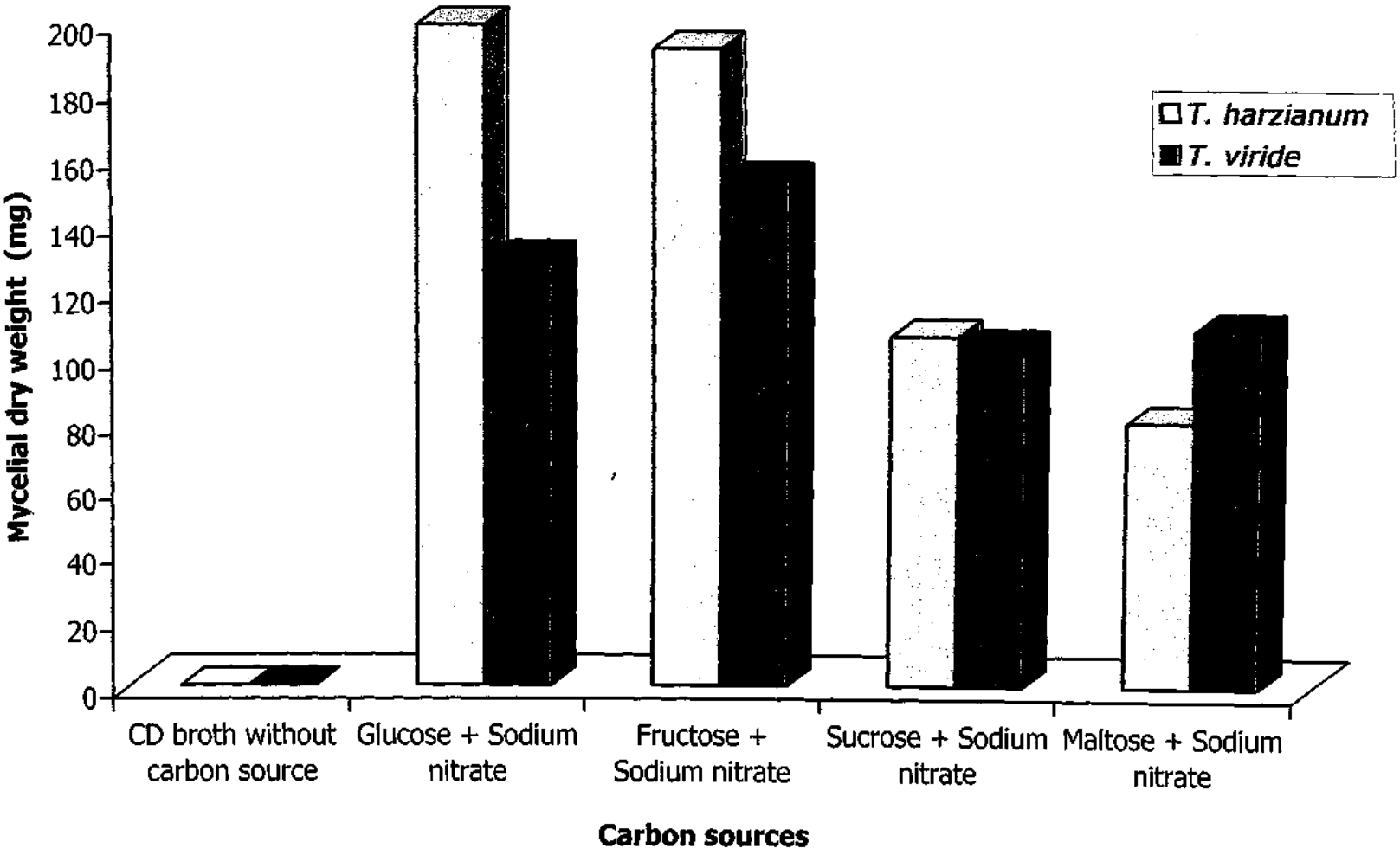


Fig. 10 Effect of different carbon sources on the growth of *Trichoderma harzianum* isolate (TH-1) and *T. viride*

carbon sources in *T. harzianum* and *T. viride* (i.e. 199.2 mg and 169.3 mg/100 ml of broth) followed by fructose that resulted in dry weight of mycelium 192.1 and 153.0 mg. Significantly less growth was found in the maltose as carbon source in both *Trichoderma* spp.

d. **Effect of Nitrogen sources:-** Urea, Potassium nitrate, Ammonium sulphate, Ammonium nitrate.

The growth (taken in terms of dry weight of mycelium) varied considerably with the nitrogen source. Urea, significantly decreased the growth of both *Trichoderma* spp. As compared to other nitrogen-sources (Table 13). Growth of both spp. Of *Trichoderma* was maximum with Ammonium sulphate dry weight (149.1 mg and 196.1 mg) of dry weight/100 ml of broth. Growth of *T. viride* was maximum with ammonium nitrate (dry weight 103.3 mg).

Above results revealed that *Trichoderma* culture raised on glucose showed more growth followed by fructose. This is because glucose is the easily available and convertible source for the *Trichoderma* strains followed by fructose. Monga (2001) reported similar results. He studied the nutritional requirements of *Trichoderma* and *Gliocladium* and reported that glucose as carbon source significantly affected the sporulation and growth of *Trichoderma* strains. He observed poor sporulation in *Trichoderma* with all carbon sources. However, he observed excellent sporulation in *Gliocladium* with all carbon sources except maltose. He reported Potassium nitrate as the best N source in case of *T. harzianum* (dry weight 309 mg). In case of *T. viride,* again more growth was

Table 13: Effect of nitrogen sources on the growth of *Trichoderma harzianum* isolate (TH-1) and *T. viride* in *in vitro* conditions using Czapeck dox broth (temperature 28±1°C and incubation period 7 days). Data represent mean of 4 replications.

Nitrogen sources		Mycelial dry weight (mg) / 100 ml broth	
		T. harzianum	*T. viride*
CD broth without N source		-	-
Urea	+ Glucose	42.2	46.2
Potassium nitrate	+ Glucose	119.5	192.0
Ammonium sulphate	+ Glucose	149.1	196.0
Ammonium nitrate	+ Glucose	136.3	103.3
C.D at 5% level		1.813	1.711

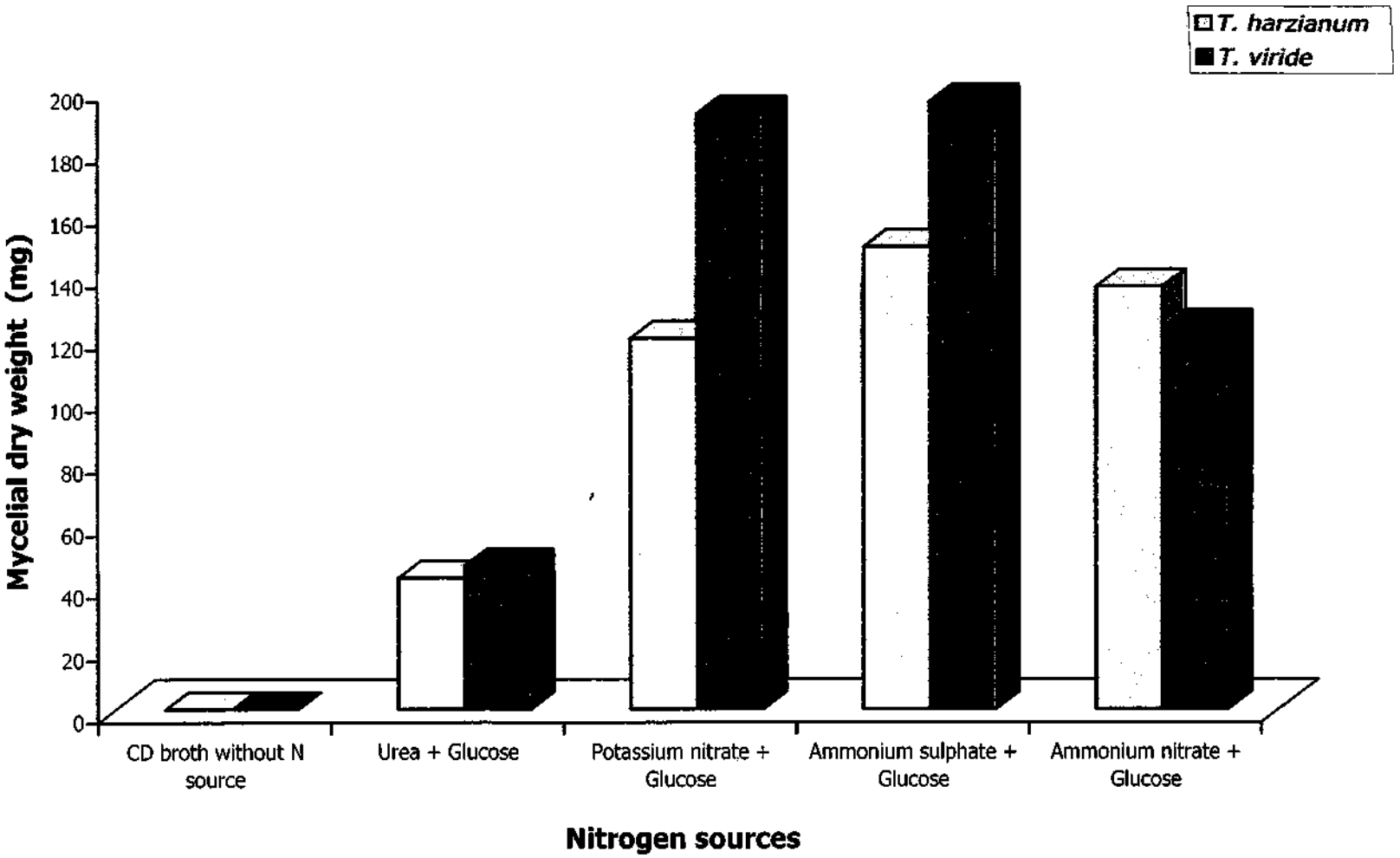


Fig. 11 Effect of different nitrogen sources on the growth of *Trichoderma harzianum* isolate (TH-1) and *T. viride*

obtained with Potassium nitrate (249 mg) as compare to Ammonium chloride (235 mg).

4.4 CHITINASE ACTIVITY OF *T. HARZIANUM* ISOLATES

4.4.1 Characterization of chitinase

Cell free extracts were subjected to polyacrylamide Disc Gel Electrophoresis (PAGE). Chitinase enzyme proteins were identified by reacting with specific substrates. The gels were removed from the tubes and incubated in 0.02M Tris-SO_4 buffer (pH 7.6) containing specific substrate at a concentration of 200 µg/ml for 30 minutes at 28±2°C followed by reaction with silver nitrate which resulted in silver chloride precipitation indicated by the formation of bands at the site of reaction on the gels.

An *in vitro* experiment was conducted to measure chitinase activity of four isolates of *T. harzianum* and one isolate of *T. viride* using chitin as a carbon source. (Table – 14) The enzyme activity was measured as ICU = Release of 1 µ mol N – Acetyl glucosamine/ml of culture filtrate/minute.

T. harzianum-4 (TH-4) showed significantly more chitinase activity of (139.23 CU) than other isolates. *Trichoderma* isolates TH-2 and TH-3 gave chitinase activity (70.29 CU and 68.71 CU respectively). *T. viride* isolate showed less chitinase activity (33.46 CU). Significantly least chitinase activity was shown by TH-1 isolate (30.51 CU). *Trichoderma harzianum* and *T. viride* act as a mycoparasite for number of fungal plant pathogens due to production of cellulase, chitinase, xylanase, lipase and protease enzymes (Chet 1990 and Harman *et al*

Table 14: Measurement of chitinase activity of *T.viride* and four isolates of *T. harzianum*. Data represent average of 5 replications.

Test sample (*T.harzianum* and *T.viride*)	Concentration of NAG O.Ds./O.Dt = Cs/Ct 0.144	Concentration in μ mol=$\frac{\text{Conc.x}10^6}{\text{M.wt}}$	Concentration in μ mol/min
TH-1	0.136	610.2	30.51
TH-2	0.371	1405.9	70.29
TH-3	0.304	1374.2	68.71
TH-4	0.616	2784.6	139.23
TV	0.148	669.2	33.46
C.D (5%)	0.182	1.62	1.81

TH : *Trichoderma harzianum*
TV : *Trichoderma viride*

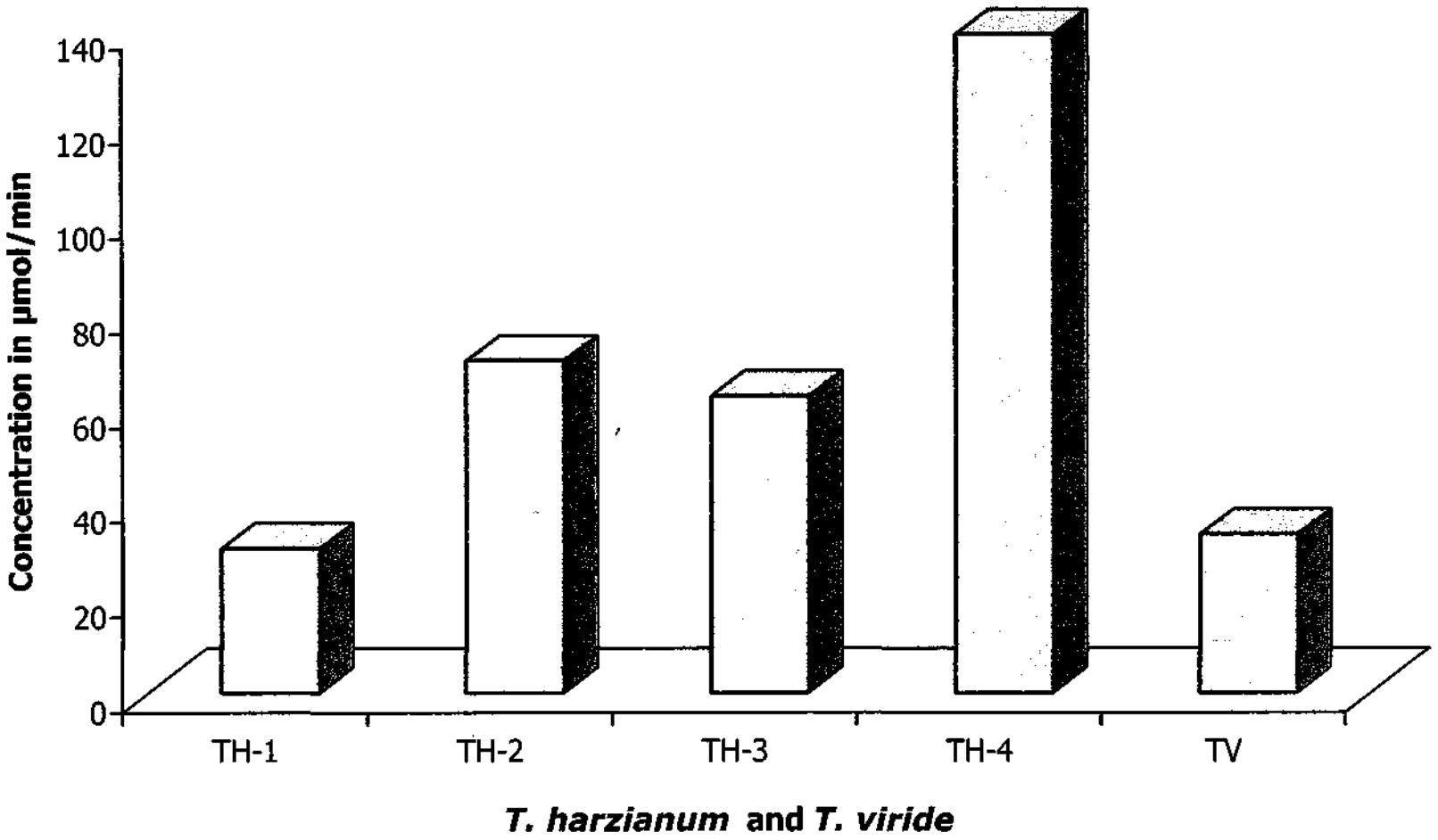


Fig. 12 Chitinase activity of *T.viride* and four isolates of *T. harzianum* (TH-1-TH-4)

1993). These enzymes are implicated in the degradation of fungal cell wall (Elad *et al* 1982). The Chitinase activity of 4 isolates of *T. harzianum* ranged from 30.51 CU to 139.23 CU. The variation in the enzyme activity clearly indicate the varying degree of substrate utilization which in turn indicate their different mycoparasitic potential. Kumar and Gupta (1999) assayed enzyme activity from culture filtrate of *T. viride* biotypes using *M. phaseolina*. All the biotypes showed a wide variation in the expression of β 1-3, glucanase and chitinase activity.

4.5 EFFECT OF CULTURE FILTRATE OF DIFFERENT ISOLATES OF *T. HARZIANUM* ON SEED GERMINATION OF SHAFTAL CROP (*TRIFOLIUM RESUPINATUM* L.) VARIETY SH – 69

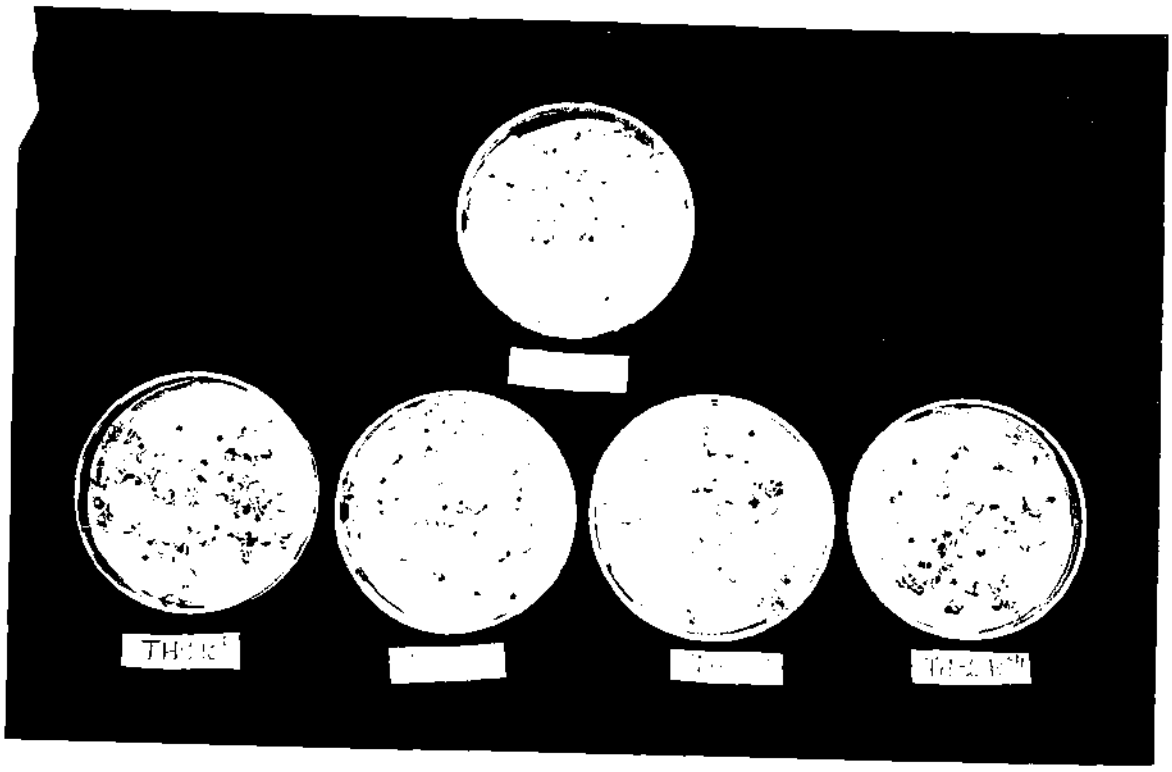
In vitro experiment was conducted to study the effect of culture filtrate of *T. harzianum* and *T. viride* on seed germination and other growth characteristics of Shaftal (*Trifolium resupinatum* L.) variety Sh – 69 (Plate 8 and 9).

The results showed 100% germination of seeds in all the treatments but seeds treated with culture filtrate germinated one day earlier than untreated control. Seeds treated with all the four isolates of *T. harzianum* and one of *T. viride* showed significant increase in the root length over untreated control isolate (Table 15). But all the treatments differs with each other regarding increase in root length (Plates 10 and 11). Seeds treated with *T. harzianum*–1 showed significantly maximum root length (4.02 cm).

Fresh weight and dry weight of seedlings also increased significantly due to seed treatment with culture filtrate of different isolates of *T. harzianum* and

Plate 8 : Effect of culture filtrate (10^{-1} - 10^{-4} dilutions) of *Trichoderma harzianum* on Shaftal (*Trifolium resupinatum*) seeds

Plate 9 : Effect of culture filtrate (10^{-1} - 10^{-4} dilutions) of *Trichoderma viride* on Shaftal (*Trifolium resupinatum*) seeds



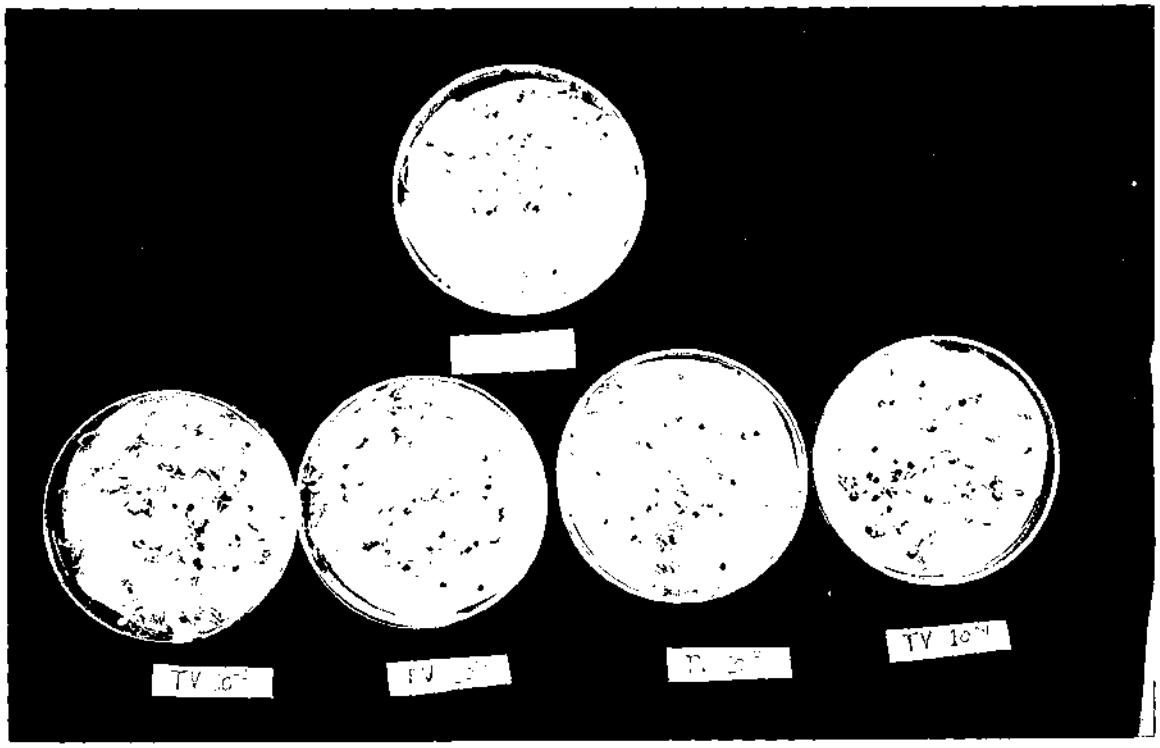
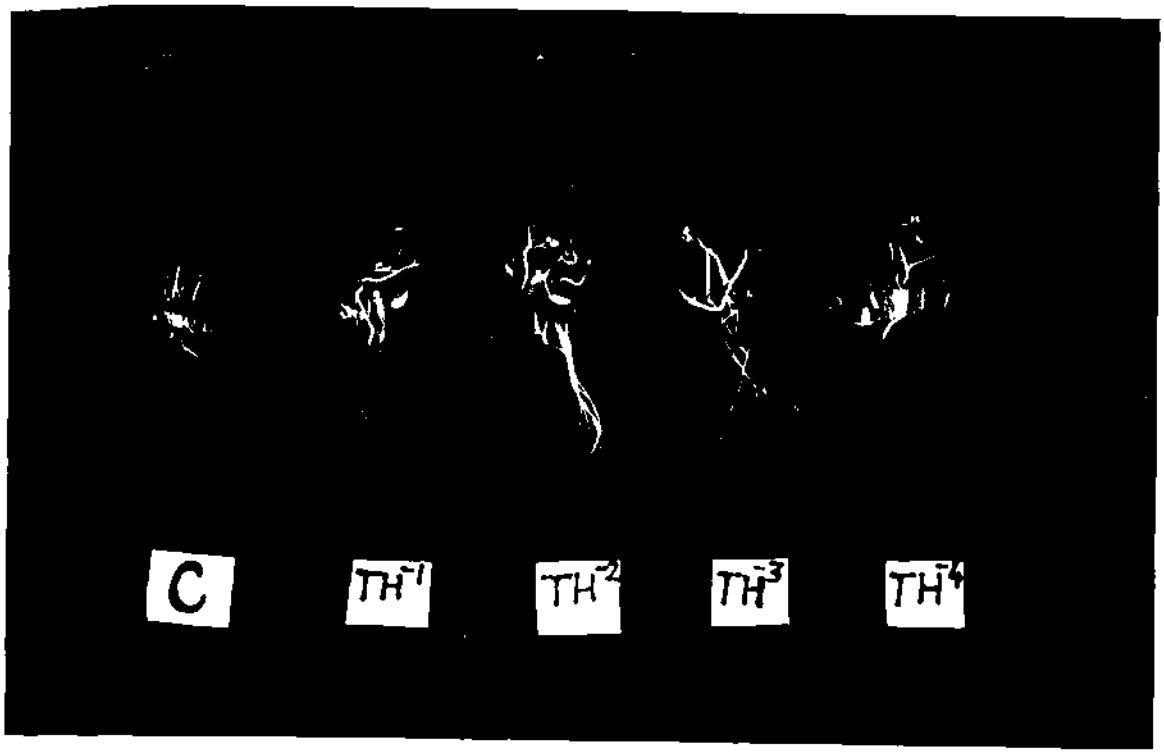

TV 10⁻⁴
TV 10⁻⁵
TV 10⁻⁶

Plate 10: Effect of culture filtrate (10^{-1} - 10^{-4} dilutions) of *Trichoderma harzianum* on root length of Shaftal (*Trifolium resupinatum*) variety Sh-69

Plate 11: Effect of culture filtrate (10^{-1} - 10^{-4} dilutions) of *Trichoderma viride* on root length of Shaftal (*Trifolium resupinatum*) variety Sh-69


C
TH1
TH2
TH3
TH4

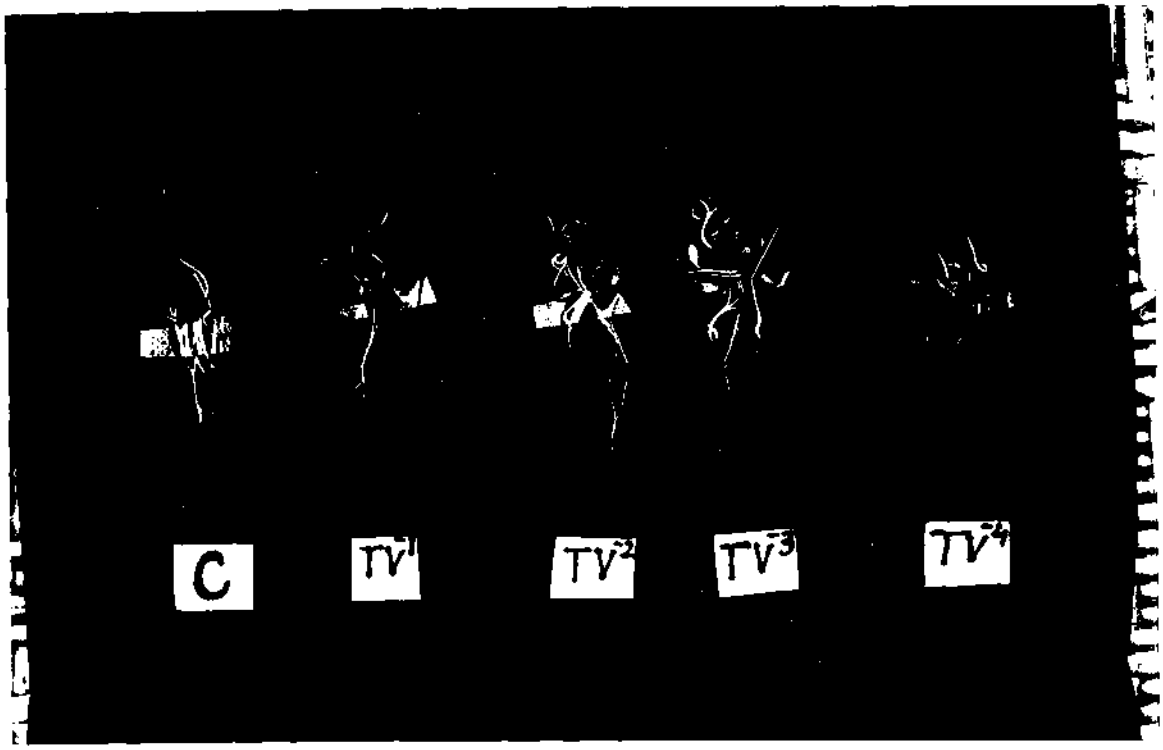

C
TV1
TV2
TV3
TV4

Table 15: Effect of culture filtrate (10^{-1}-10^{-4} dilutions) of *Trichoderma spp.* on seed germination and other growth characters of Shaftal variety Sh-69. Data represent average of 4 replications.

Treatments	Germination (%)	Root length (cm)				Fresh weight of seedlings (mg)				Dry weight of seedlings (mg)			
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Control	100	3.017				19.34				7.24			
T. harzianum-1	100	4.02	3.72	3.22	3.11	31.24	29.36	25.42	22.44	10.02	9.36	8.34	7.22
T. harzianum-2	100	3.61	3.44	3.21	3.20	29.36	27.22	20.34	20.04	9.37	9.26	6.90	6.72
T. harzianum-3	100	3.73	3.71	3.56	3.33	30.34	28.32	21.22	20.36	9.34	8.36	7.62	7.44
T. harzianum-4	100	3.67	3.63	3.49	3.40	27.21	26.42	20.13	20.31	9.32	7.46	7.39	6.33
T. viride	100	3.48	3.21	3.17	3.01	26.30	22.42	21.33	21.04	8.36	7.44	6.39	6.32
CD at 5% level					0.337				NS				NS

◆ Superscripts indicate dilutions of culture filtrate

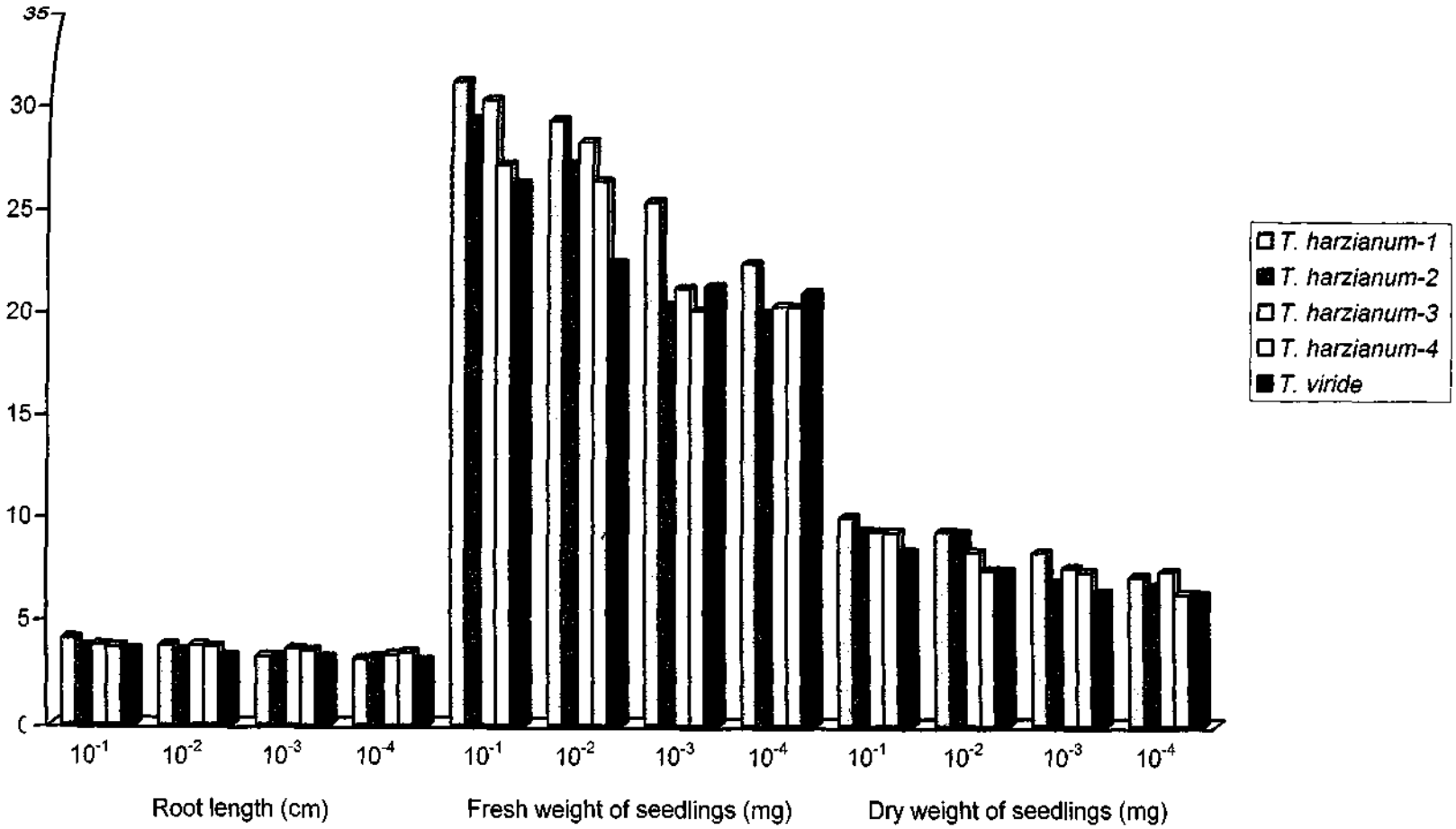


Fig. 13 Effect of culture filtrate (10^{-1} - 10^{-4} dilutions) of *Trichoderma spp.* on seed germination and other growth characters of Shaftal variety Sh-69

a isolates of *T. viride. T. harzianum*–1 showed the maximum fresh weight and dry weight of seedlings (31.24 mg and 10.02 mg at dilution 10^{-1}) respectively. Treatment of seeds with culture filtrate of all the 4 isolates showed significant increase in fresh and dry weights of seedlings.

Dilutions of culture filtrate (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of *Trichoderma* spp. showed positive effect on root length, fresh and dry weights of seedlings. Further, it has been concluded that dilutions of culture filtrate of all the four *T. harzianum* isolates and one isolate of *T. viride* decreased beneficial effect.

Effect of culture filtrate of *Trichoderm spp.* on *Rhizobium* was also observed (Table 16 and 17). There was no adverse effect of culture filtrate of *Trichoderma* on *Rhizobium.* The results obtained were in line with the report of Wu (1982) that seeds treated with *Trichoderma pseudokoningii* – 4 and 5% methyl cellulose suspension for 30 minutes showed significantly better results in the emergence of seeds than the untreated seeds. Likewise, Chang *et al* (1986) recorded increased plant growth with *T. harzianum*. They concluded that in raw soil containing the fungus, pepper seeds germinated two days earlier than the untreated control. Kleifeld and Chet (1992) studied the interaction of *Trichoderma harzianum* with plants. They reported increased emergence of seedlings, plant height, leaf area and dry weight of moongbeans, tomatoes, radishes, chillies and cucumber. Bae *et al* (1995) reported similar results. The beneficial effect of *T. harzianum* may be attributed to the production of growth regulators (Skrobakova 1995; Shivana *et al* 1994; 1996; Altomare *et al* 1999). Altomare *et al* (1999)

Table 16: Effect of culture filtrate of *Trichoderma spp* on growth of *R. leguminosarum* bv *trifolii* isolate(R-1). Data represent mean of 5 replications.

Treatments YEM broth + *Rhizobium*+Culture filtrate of *T.harzianum*				*T.harzianum* O.D	*T.viride* O.D
100ml	+ 2ml	+	-	0.135	0.135
98ml	+ 2ml	+	2ml	0.179	0.157
96ml	+ 2ml	+	4ml	0.191	0.178
94ml	+ 2ml	+	6ml	0.199	0.179
92ml	+ 2ml	+	8ml	0.208	0.168
90ml	+ 2ml	+	10ml	0.222	0.162
Culture filtrate				0	0

Table 17: Effect of culture filtrate *T. harzianum* isolate (TH-1) on growth and morphology of *R. leguminosarum* bv *trifolii* isolate(R-1). Data represent mean of 5 replications.

Treatments YEM broth + *Rhizobium*+Culture Filtrate of *T. harzianum*				pH	O.D	Changes in *Rhizobium* morphology	Viscosity
100ml	+ 2ml	+	-	7.0	0.135	Rod shaped	Viscous
98ml	+ 2ml	+	2ml	6.7	0.179	Rod shaped	Viscous
96ml	+ 2ml	+	4ml	6.7	0.191	Rod shaped	Viscous
94ml	+ 2ml	+	6ml	6.6	0.199	Rod shaped	Less Viscous
92ml	+ 2ml	+	8ml	6.3	0.208	Rod shaped	Less Viscous
90ml	+ 2ml	+	10ml	6.2	0.222	Rod shaped	Less Viscous

documented that the capability of *T. harzianum* Rifai 1295-22, T-22 to solubilize minerals via three possible mechanisms i.e. acidification of the medium, production of the chelating metabolites and redox acitivity.

4.6 EFFECT OF EFFICIENT ISOLATE OF *R. LEGUMINOSARUM* BV. *TRIFOLII* AND *T. HARZIANUM* INDIVIDUALLY AND IN COMBINATION ON VARIOUS GROWTH CHARACTERISTICS AND NITROGEN FIXATION IN SHAFTAL (*TRIFOLIUM RESUPINATUM* L.) VARIETY SH-69 UNDER FIELD CONDITIONS

A field trial was conducted to examine the effect of efficient isolate of *R. leguminosarum* bv. *trifolii* and isolate of *T. harzianum* individually and in combination on various growth characters in Shaftal ((*Trifolium resupinatum* L.) variety Sh-69 under field conditions. Efficient isolate of *Rhizobium leguminosarum*, an isolate of *T. harzianum* and *T. viride* (carrier based) were used for the experiment. Eight treatments were kept with four replications each. Three cuttings have been taken and following observations were recorded.

4.6.1 First cutting

First cutting was taken after 55 days of sowing and following observations were recorded.

Fresh weight of shoot and root was (5.92 g/plant and 1.22 g/plant respectively) significantly more in R-1 isolate as compared to control and *Trichoderma* treatments (TH-1 and TV). The treatment having combination of R-1 and TH-1 resulted in significantly high fresh weight of shoot and root i.e. 6.96 g and 1.29 g/plant (Table 18). Less fresh weight of shoot and root was obtained in treatments containing *T. harzianum*, *T. viride* alone and in combination TH-1+TV

Table 18: Effect of efficient isolates of *R. leguminosarum* bv *trifolii* (R-1) and *Trichoderma* spp. on various growth characters of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under field conditions (after 55 days of sowing). Data represent mean of 4 replications.

Treatments	Fresh weight of shoot (g/plant)	Fresh weight of Root (g/plant)	Dry weight of shoot (g/plant)	Dry weight of Root (mg/ plant)	Total chlorophyll contents of leaves mg/0.5g of fresh leaves	Nitrate reductase activity of leaves µM/0.5g of leaf sample
Control	4.73	1.13	1.36	26.21	1.12	0.49
R-1	5.92	1.22	1.72	29.60	1.47	0.52
TH-1	4.34	1.20	1.62	28.20	1.31	0.53
TV	4.93	1.19	1.38	27.30	1.29	0.51
R-1+TH-1	6.96	1.29	1.78	30.10	1.48	0.53
R-1+TV	6.02	1.29	1.76	31.10	1.48	0.52
TH-1+TV	5.63	1.26	1.73	28.40	1.33	0.52
R-1+TH-1+TV	6.12	1.21	1.81	32.90	1.49	0.54
C.D at 5% level	0.173	0.133	0.129	0.136	0.171	NS

R: *Rhizobium*
TH: *Trichoderma harzianum*
TV: *Trichoderma viride*

i.e. (4.34 g and 1.20 g; 4.93 g and 1.19 g and 5.63 g and 1.26 g/plant respectively) but it was a non significant effect. Dry weight of shoot and root also found to be more in *Rhizobium* isolate R-1 alone and in combination of *Rhizobium* and *T. harzianum* (R-1 + TH-1).

Total Chlorophyll contents of leaves was found to be significantly maximum in case of the treatment with combination of *Trichoderma harzianum*–1 and *Rhizobium*-1 (1.48 mg/0.5 g of fresh leaves) followed by *Rhizobium* isolate R-1 alone (1.47 mg). Significantly least chlorophyll content was observed in *T. viride* alone (1.29 mg) followed by the combination of TH-1 + TV (1.33 mg/0.5 g of fresh leaves).

Regarding nitrate reductase activity, non-significant increase was observed in all the treatments. High value of NRA activity was observed in combination of R-1 + TH-1 + TV (0.54 μM/0.5 g of leaf sample).

There is significant increase in the number of nodules/plant (Table 19). *Rhizobium* isolate (R-1) showed the maximum number of nodules/plant and their dry weight (46 nodules/plant and 24.2 mg dry weight/plant) respectively. TH-1 and TV treatment showed significantly lesser number of nodules (36 and 34 nodules) and their dry weight (19.3 mg and 18.7 mg/plant) respectively. The combination of *Rhizobium* and *Trichoderma harzianum* (R-1 + TH-1) and (R-1 + TH-1 + TV) produced higher number of nodules (49 nodules/plant).

Non-significant increase has been observed in the Leghaemoglobin content of nodules. Maximum leghaemoglobin content was observed in treatment

Table 19 : Effect of efficient isolates of *R. leguminosarum* bv *trifolii* (R-1) and *Trichoderma* spp. on various growth characters of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under field conditions (after 55 days of sowing). Data represent mean of 4 replications.

Treatments	Number of nodules/plant	Dry weight of nodules (mg/plant)	Leghaemoglobin content of nodules mg/0.5 g of nodules	Nitrogen content of shoot(%)
C	33	17.4	1.31	0.92
R-1	46	24.2	1.32	1.76
TH-1	36	19.3	1.29	1.24
TV	34	18.7	1.28	1.18
R-1 + TH-1	49	24.4	1.34	1.84
R -1 + TV	43	22.9	1.37	1.16
TH-1 + TV	35	19.5	1.30	1.01
R-1+ TH-1 + TV	49	24.3	1.39	1.78
C.D at 5% level	1.73	0.169	0.302	0.436

R : *Rhizobium*
TH : *Trichoderma harzianum*
TV : *Trichoderma viride*

R-1 + TV followed by R-1 + TH-1 i.e. (1.37 mg and 1.34 mg) respectively as compared to control (1.31 mg).

The percentage of nitrogen was found to be significantly maximum in the treatment containing R-1 + TH-1 + TV (1.78%) followed by *Rhizobium* R-1 alone (1.76%). Significantly lesser amount of nitrogen was shown by TH-1 (1.24%) and TV (1.18%) followed by R-1 + TV (1.16%).

4.6.2 Second cutting

Second cutting was taken after 80 days of sowing and following observations were recorded.

Fresh weight of shoot and root has been found to be significantly high in *Rhizobium* isolate (R-1) alone i.e. 4.93 g and 1.16 g/plant respectively (Table 20). There was slight increase in fresh weight of shoot and root in TH-1 and TV alone. However, the combination of R-1 and TH-1 resulted in significantly more fresh weight (5.86 g/plant) followed by R-1 + TV treatment. (5.78 g/plant) *Rhizobium* treatment (R-1) alone also resulted in significantly high value of dry weight of shoot and root i.e. 1.61 g and 27.42 mg respectively. Significantly maximum amount of dry weights of shoot and root were observed in combination of *Rhizobium* isolate, *T. harzianum* and *T. viride* (R-1 + TH-1 + TV) 1.72g and 31.24 mg/plant.

The treatment containing combination of R-1 + TH-1 resulted in significantly high total chlorophyll contents 1.39 mg which in turns followed by R-1 + TV 1.37 mg/0.5g of leaves. Least amount of total chlorophyll content was

Table 20 : Effect of efficient isolate of *R .leguminosarum* bv *trifolii* (R-1) and *Trichoderma* spp. on various growth characters of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under field conditions (after 80 days of sowing).Data represent mean of 4 replications.

Treatments	Fresh weight of shoot (g/plant)	Fresh weight of Root (g/plant)	Dry weight of shoot (g/plant)	Dry weight of Root (mg/ plant)	Total chlorophyll contents of leaves mg/0.5g of fresh leaves	Nitrate reductase activity of leaves µM/0.5g of leaf sample
C	3.96	1.12	1.27	25.16	1.09	0.42
R-1	4.93	1.16	1.61	27.42	1.36	0.49
TH-1	4.24	1.22	1.49	26.33	1.29	0.46
TV	4.13	1.18	1.29	28.12	1.31	0.42
R-1+TH-1	5.86	1.27	1.62	29.31	1.39	0.42
R-1+TV	5.78	1.31	1.63	30.16	1.37	0.46
TH-1+TV	5.21	1.24	1.59	29.12	1.31	0.42
R-1+TH-1+TV	6.7 3	1.29	1.72	31.24	1.42	0.44
C.D at 5% level	0.169	0.171	0.122	0.136	0.176	NS

R: Rhizobium
TH: Trichoderma harzianum
TV: Trichoderma viride

observed in TH-1 alone 1.29 mg.

NRA do not show any significant increase but maximum value was obtained from *Rhizobium* treatment (0.49 μM) alone followed by TH-1 alone and R-1 + TV treatment.

The combination of R-1 and TV significantly produced higher number of nodules and their dry weight (47 nodules/plant and 23.3 mg/plant respectively) followed by R-1 + TH-1 and R-1 + TH-1 + TV showed 39 nodules/plant each and dry weight is 22.1 mg and 22.9 mg/plant respectively.

The leghaemoglobin content was significantly more in the treatment R-1 + TH-1 + TV as compared to other treatments (1.29 mg). The treatment R-1 + TV showed the higher leghaemoglobin content as compared to R-1 + TH-1 i.e. 1.24 mg and 1.23 mg respectively (Table 21). Lesser amount of leghaemoglobin content was shown by TV and TH-1 (1.19 mg and 1.17 mg) respectively.

There was significant increase in the percentage of nitrogen content (1.79%) in treatment R-1 followed by R-1 + TH-1 (1.78%) significantly less amount of nitrogen was shown by treatment TH-1 alone (1.20%) and TH-1 + TV (1.12%).

4.6.3 Third cutting

The third cutting was taken after 110 days of sowing and following observations were taken.

There was significantly more fresh weight of shoot and root (Table 22) was produced by the treatment containing the combination of R-1+TH-1+TV.

Table 21: Effect of efficient isolates of *R. leguminosarum* bv *trifolii* (R-1) and *Trichoderma* spp. on various growth characters of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under field conditions (after 80 days of sowing). Data represent mean of 4 replications.

Treatments	Number of nodules /plant	Dry weight of nodules (mg/plant)	Leghaemoglobin Content of nodules mg/0.5g of nodules	Nitrogen content of shoot(%)
C	29	16.6	1.19	0.92
R-1	32	21.2	1.21	1.79
TH-1	33	17.6	1.17	1.20
TV	37	19.2	1.19	1.63
R-1 + TH-1	39	22.1	1.23	1.78
R-1 + TV	47	23.3	1.24	1.51
TH-1 + TV	32	20.1	1.26	1.12
R-1+ TH-1 + TV	39	22.9	1.29	1.61
C.D at 5 % level	1.32	0.176	0.282	0.463

R: *Rhizobium*
TH: *Trichoderma harzianum*
TV: *Trichoderma viride*

Table 22: Effect of efficient isolates of *R. leguminosarum* bv *trifolii* (R-1) and *Trichoderma* spp. on various growth characters of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under field conditions (after 110 days of sowing). Data reveals mean of 4 replications.

Treatments	Fresh weight of shoot (g/plant)	Fresh weight of Root (g/plant)	Dry weight of shoot (g/plant)	Dry weight of Root (mg/plant)	Total chlorophyll contents of leaves mg/0.5g of fresh leaves	Nitrate reductase activity of leaves µM/0.5g of leaf sample
C	3.78	1.10	1.29	24.16	1.11	0.49
R-1	4.66	1.18	1.59	28.14	1.32	0.51
TH-1	4.13	1.23	1.61	26.13	1.26	0.48
TV	4.39	1.26	1.31	25.11	1.30	0.42
R-1 + TH-1	4.99	1.29	1.66	29.61	1.33	0.50
R-1 + TV	5.05	1.29	1.62	30.11	1.34	0.54
TH-1 + TV	4.99	1.31	1.64	29.19	1.30	0.52
R-1+ TH-1 + TV	5.63	1.30	1.71	30.18	1.36	0.51
C.D at 5 % level	0.405	0.632	0.172	0.136	0.877	NS

TH: *Trichoderma harzianum*
TV: *Trichoderma viride*

(5.63 g and 1.30 g respectively) as compared to control and other treatments. The treatment R-1 + TV gave high fresh weight of shoot and root followed by TH-1 + TV i.e. (5.05 g and 1.29 g) followed by (4.99 g and 1.31 g respectively). Significantly less amount of fresh and dry weight was produced by TV and TH-1 alone (4.39 g and 1.26 g; 4.13 and 1.23 g respectively). Significantly higher amount of dry weight was produced by treatment R-1 + TH-1 + TV (1.71 mg and 30.18 mg) followed by R-1 + TH-1 (1.66 mg and 29.61 mg respectively).

Similar trend has been observed in the total chlorophyll contents. There was significantly higher values in the treatments R-1 + TH-1 + TV followed by R-1 + TH-1 (1.36 mg and 1.33 mg) respectively. Significantly lesser amount of chlorophyll contents was produced by TV and TH-1 alone (1.30 mg and 1.26 mg) respectively.

The nitrate reductase activity has shown a non-significant increase. Maximum NRA was observed in treatment R-1 + TV and TH-1+TV (0.54 μM and 0.52 μM) respectively.

Number of nodules and their dry weight varies with treatment R-1 + TH-1 + TV (Table 23) that produced higher amount of nodules and their dry weight (38.5 nodules and 14.16 mg respectively) as compared to other treatments. Significantly lesser amount of nodules and their dry weight was produced by TH-1 and TV alone (28.6 and 10.14 mg; 27.2 and 10.12 mg) respectively.

The treatment containing combination of R-1 + TH-1 + TV significantly produced higher amount of leghaemoglobin content 1.36 mg. There

Table 23: Effect of efficient isolates of *R. leguminosarum* bv *trifolii* (R-1) and *Trichoderma* spp. on various growth characters of Shaftal (*Trifolium resupinatum* L.) variety Sh-69 under field conditions (after 110 days of sowing). Data represent mean of 4 replications.

Treatments	Number of nodules/plant	Dry weight of nodules (mg/plant)	Leghaemoglobin content of nodules mg/0.5g of nodules	Nitrogen content of shoot(%)
C	26.3	10.06	1.29	0.84
R-1	31.5	11.12	1.33	1.64
TH-1	28.6	10.14	1.31	1.50
TV	27.2	10.12	1.34	1.48
R-1 + TH-1	32.2	13.12	1.31	1.59
R-1 + TV	31.1	11.16	1.30	1.57
TH -1+ TV	36.8	12.19	1.31	1.23
R-1+ TH-1 + TV	38.5	14.16	1.36	1.59
C.D. at 5% level	0.182	0.165	0.173	0.464

R : *Rhizobium*
TH : *Trichoderma harzianum*
TV : *Trichoderma viride*

was non-significant increase in leghaemoglobin content in the treatment R-1 + TV and TH-1 alone (1.30 mg and 1.31 mg) respectively.

The percentage of Nitrogen was found to be significantly high in the treatment R-1 alone (1.64%) followed by R-1 + TH-1 and R-1 + TH-1 + TV (1.59%). Significantly lesser amount of nitrogen % was shown by TH-1 + TV (1.23%).

Three cuttings have been taken after 55, 80 and 110 days respectively (Table 24). There was non-significant increase in the green fodder yield (Kg/plot). Maximum total green fodder yield was obtained in the combination treatment R-1+TH-1 (81.9 Kg/plot) followed by TH-1 (81.4 Kg/plot). Similarly, maximum plant height (Table 25) was obtained in the combination of R-1+TH-1+TV (66.4 cm) followed by R-1+TH-1 (65.9 cm)

Perusal of Data (Table 18-25) revealed that under field conditions large variation occurred in the efficiency of *R. leguminosarum* bv *trifolii* and *T. harzianum*. It has been observed that *Rhizobium* alone affected fresh and dry weights of shoot and root. When *Trichoderma* was used alone there was less increase in all growth characters but when it was used in combination with *Rhizobium* isolate (R–1) significant increase in all growth characters was observed. The yield (kg/plot) obtained was significantly high in the treatments containing *Rhizobium* and *Trichoderma* together. This depicts that combination of *Rhizobium* and *Trichoderma* proved to be better than other treatments. Likewise, Jayaraj and Ramabadran (1999) conducted an experiment and reported non-

Table 24: Total green fodder yield of Shaftal (*Trifolium resupinatum* L.) in Kg/plot after 3 cuttings under field conditions

Treatments	First Cutting (55 Days)	Second cutting (80 Days)	Third Cutting (110 Days)	Total green fodder yield
C	28.5	27.4	24.7	80.6
R – 1	31.1	25.7	24.3	81.1
TH – 1	30.9	25.6	24.9	81.4
TV	29.8	25.9	25.6	81.3
R –1 + TH – 1	30.9	25.9	25.1	81.9
R – 1 + TV	31.9	24.2	25.2	81.3
TH – 1 + TV	30.5	24.6	25.7	80.8
R – 1 + TH + TV	32.6	24.1	24.6	81.3
C.D at 5% level				NS

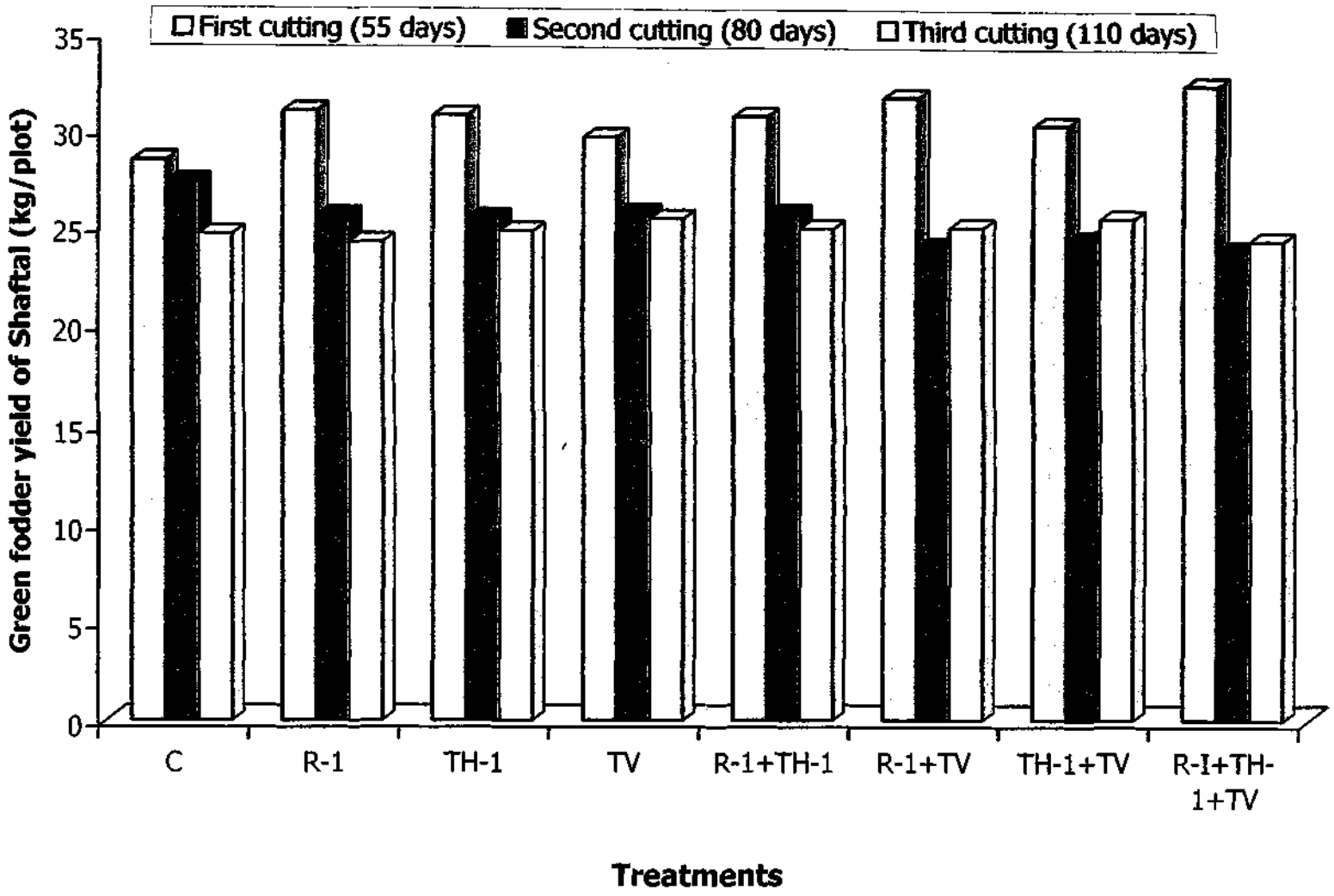


Fig. 14 Total green fodder yield of Shaftal (*Trifolium resupinatum* L.) in kg/plot under field conditions

Table 25: Height (cm) and total green fodder yield (Kg/ plot) of Shaftal (*Trifolium resupinatum* L.) under field conditions

Treatments	Plant height (cm)	Total green fodder yield Kg/plot
C	61.3	80.6
R – 1	62.5	81.1
TH – 1	65.6	81.4
TV	64.3	81.3
R –1 + TH – 1	65.9	81.9
R – 1 + TV	64.8	81.3
TH – 1 + TV	65.5	80.8
R – 1 + TH + TV	66.4	81.3

♦ Representative samples were taken for measuring plant height.

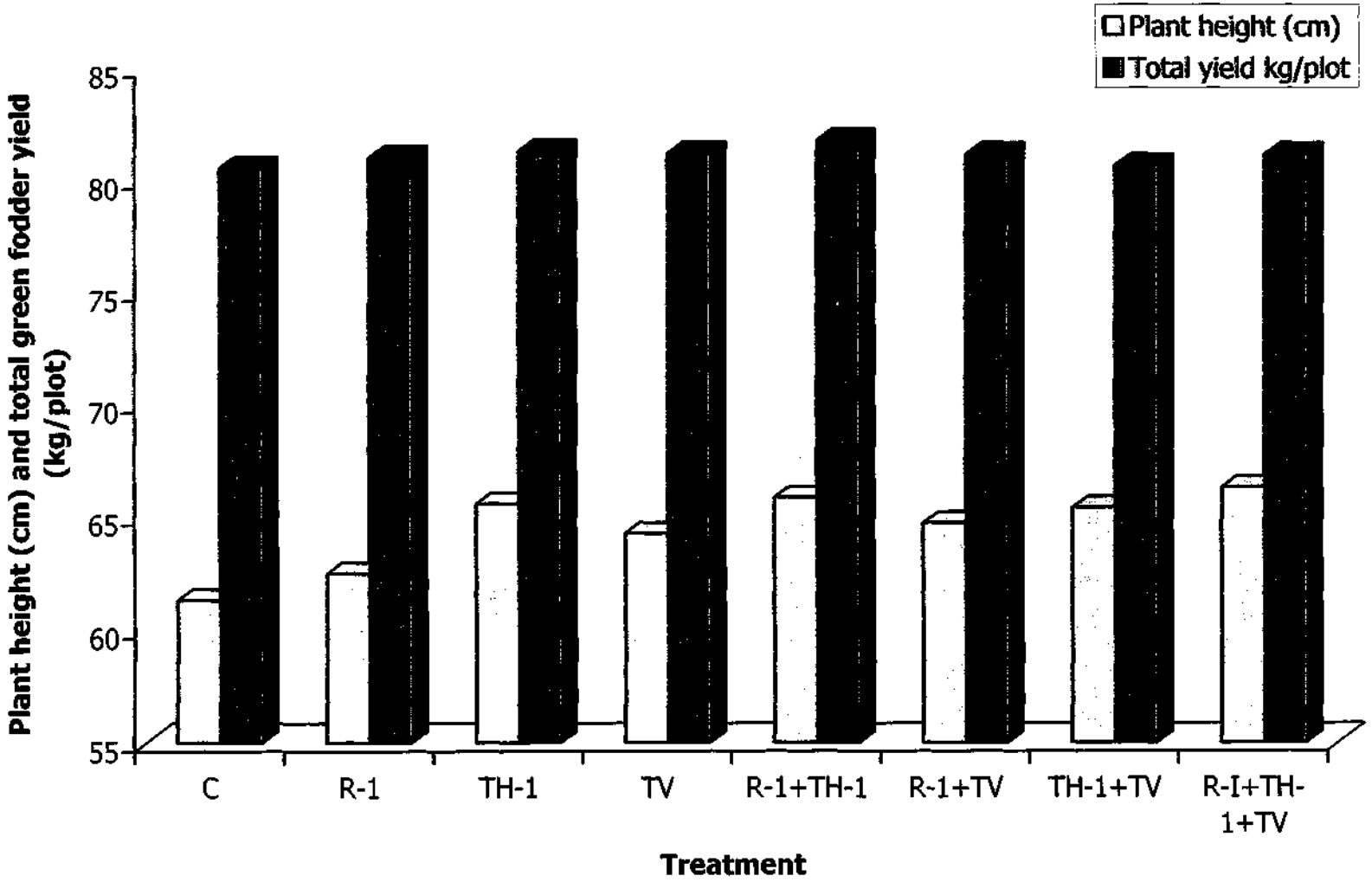


Fig. 15 Height (cm) and total green fodder yield (kg/plot) of Shaftal (*Trifolium resupinatum* L.) under field conditions

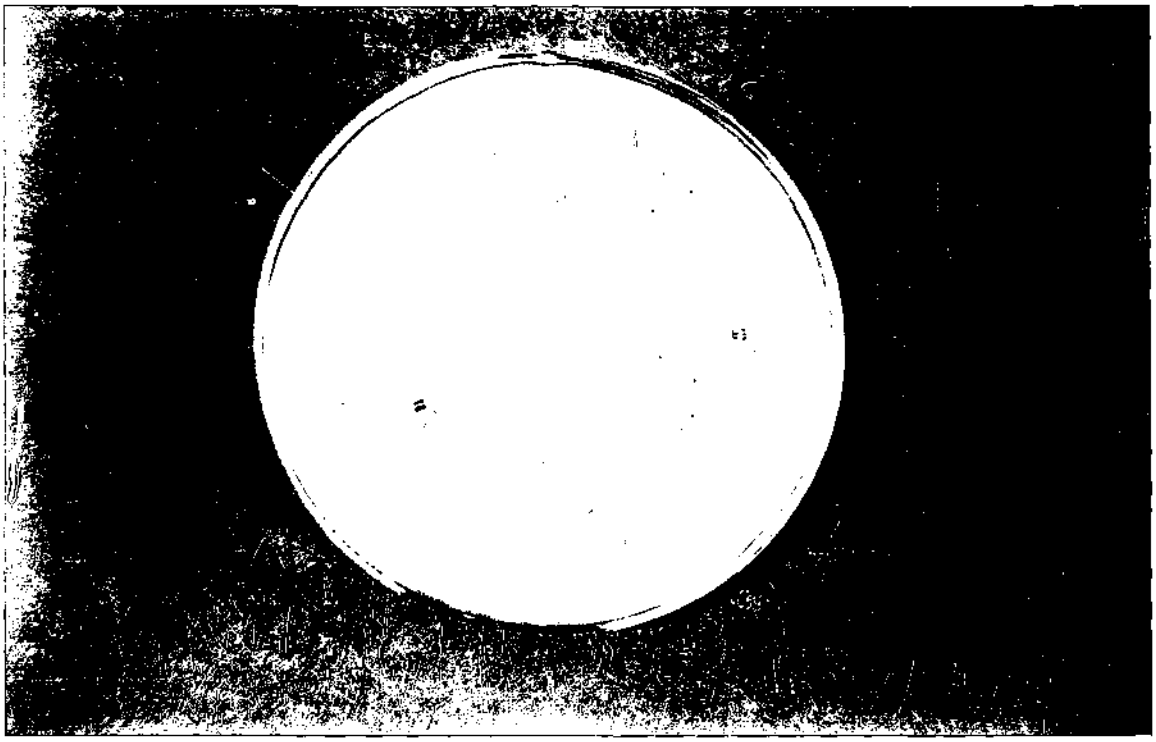
significant increase in nodulation between *Rhizobium* alone and *Rhizobium* + *Trichoderma* treatments. Though a significant increase in the biomass yield was observed with the latter. However, in the field experiment a slight increase in the seed yield was recorded (37.66%) but again no significant difference in number of nodules between *Rhizobium* alone and *Rhizobium* + *Trichoderma* could be recorded. Interestingly, a significant reduction in the incidence of root rot in *Rhizobium* + *Trichoderma* treatment was observed when compared with *Trichoderma* alone. The seed yield and total biomass yield increased slightly in *Rhizobium* + *Trichoderma* treatment when compared with others.

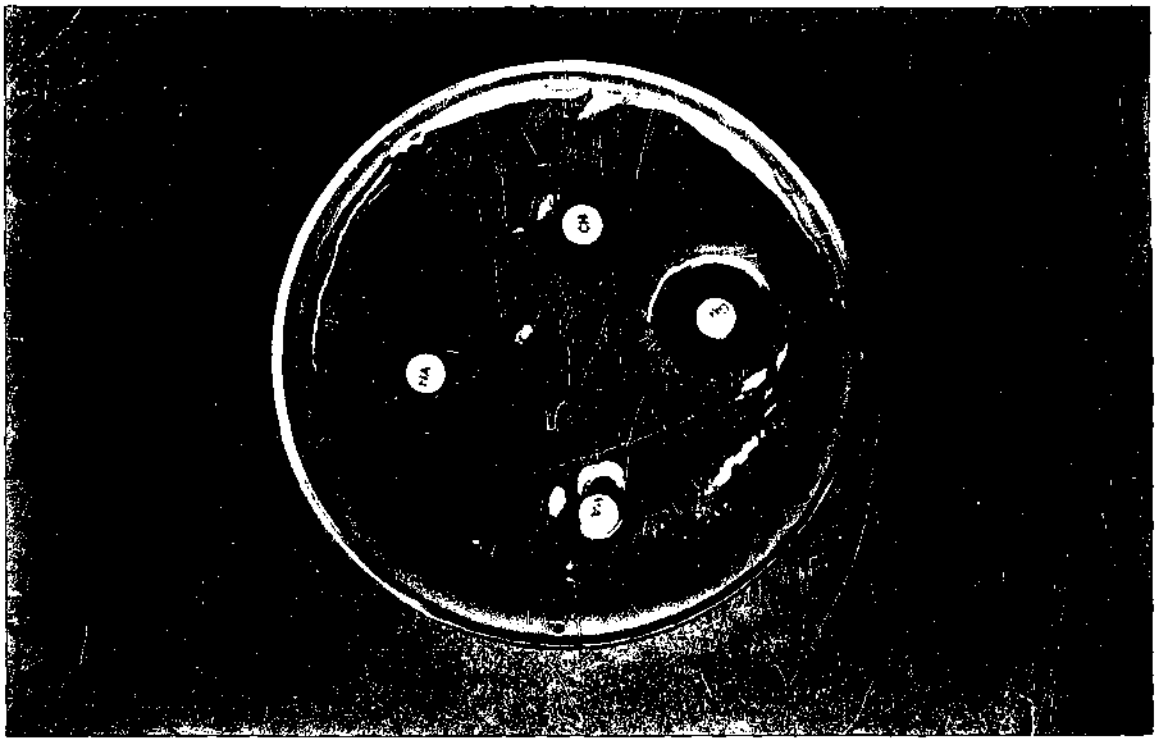
4.7 COMPETITIVENESS OF *R. LEGUMINOSARUM* BV. *TRIFOLII* AND ESTABLISHMENT OF *T. HARZIANUM* IN *RHIZOSPHERE* OF SHAFTAL CROP (*TRIFOLIUM RESUPINATUM* L.) VARIETY SH – 69

Selected antibiotics viz. Erythromycin (ER) 15 mcg, Tetracycline (TE) 30 mcg, Kanamycin (KA) 10 mcg, Gentamycin (GM) 10 mcg, Vancomycin (VA) 30 mcg, Streptomycin (SM) 10 mcg, Nalidixic acid (NA) 30 mcg and Chloramphenicol (CH) 30 mcg were used to study competitiveness of inoculated strain R-1 (Plate 12 and 13). The competitiveness of strain R-1 showed 51.26 per cent nodulation. Scientists in the past have also studied competitiveness of inoculated strain of *Rhizobium* in case of different legume crops (Josey *et al* 1979, Chahal *et al* 1982 and Bassam and Gresshoff 1986).

Establishment of the antagonist *Trichoderma harzianum* was observed in the rhizosphere of Shaftal plant by dilution plate count. The plate

Plate 12 & 13 : Inhibition zones shown by *Rhizobium* isolate (R-1)





count of the freshly prepared inoculation of *Trichoderma harzianum* in a carrier based culture gave 41 colony forming units/g of inoculum i.e in 10 g there were 410 units of the organism.

4.8 SCREENING OF SUBSTRATES FOR MULTIPLICATION OF *T. HARZIANUM*

An *in vitro* experiment was conducted to screen the substrates for multiplication of *T. harzianum* and *T. viride* (Table 26 and Table 27) respectively. For this, substrates i.e. wheat straw, wheat bran, rice straw, sugarcane bagasse, potato peels, boiled potato, fruit juice waste (orange), fruit peel (orange), tea leaves waste and compost were used (Plate 14). The results indicated that the wheat bran was the most suitable substrate for the mass production of *T. harzianum* (49.2 $x10^4$ CFU/g). Ten days after incubation in sterilized substrates, the population of *T. harzianum* and *T. viride* on wheat bran showed significantly more CFU/g than other substrates. A few workers in the past tried to find cheap substrates for multiplication of *T. harzianum* and found wheat bran as the better substrate. (Shamarao *et al* 1998; Prasad and Rangeshwaran 2000 and Gandhi Kumar *et al* 2001).

Plate 14: Growth of *Trichoderma harzianum* isolate (TH-1) on various substrates

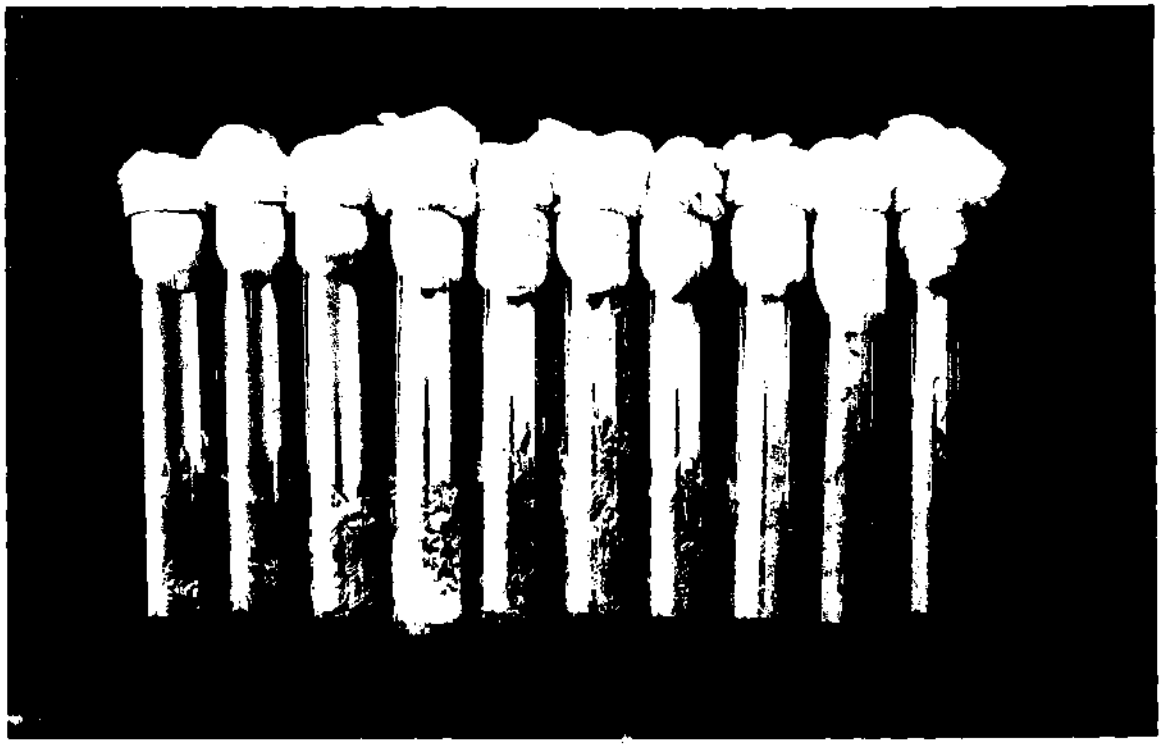


Table 26 : Effect of different substrates on the growth of *T. harzianum* isolate (TH-1). Data represent mean of 4 replications.

Substrates	CFU/g
Wheat straw	$29.1x10^4$
Wheat bran	$49.2 x10^4$
Rice straw	$26.1 x10^4$
Sugarcane bagasse	$47.3 x10^4$
Potato peels	$42.4 x10^4$
Boiled potato	$45.6 x10^4$
Fruit juice waste (citrus)	$42.2 x10^4$
Fruit peels (citrus)	$39.3 x10^4$
Tea leaves waste	$37.6 x10^4$
Compost	-
CD at 5% level	0.156

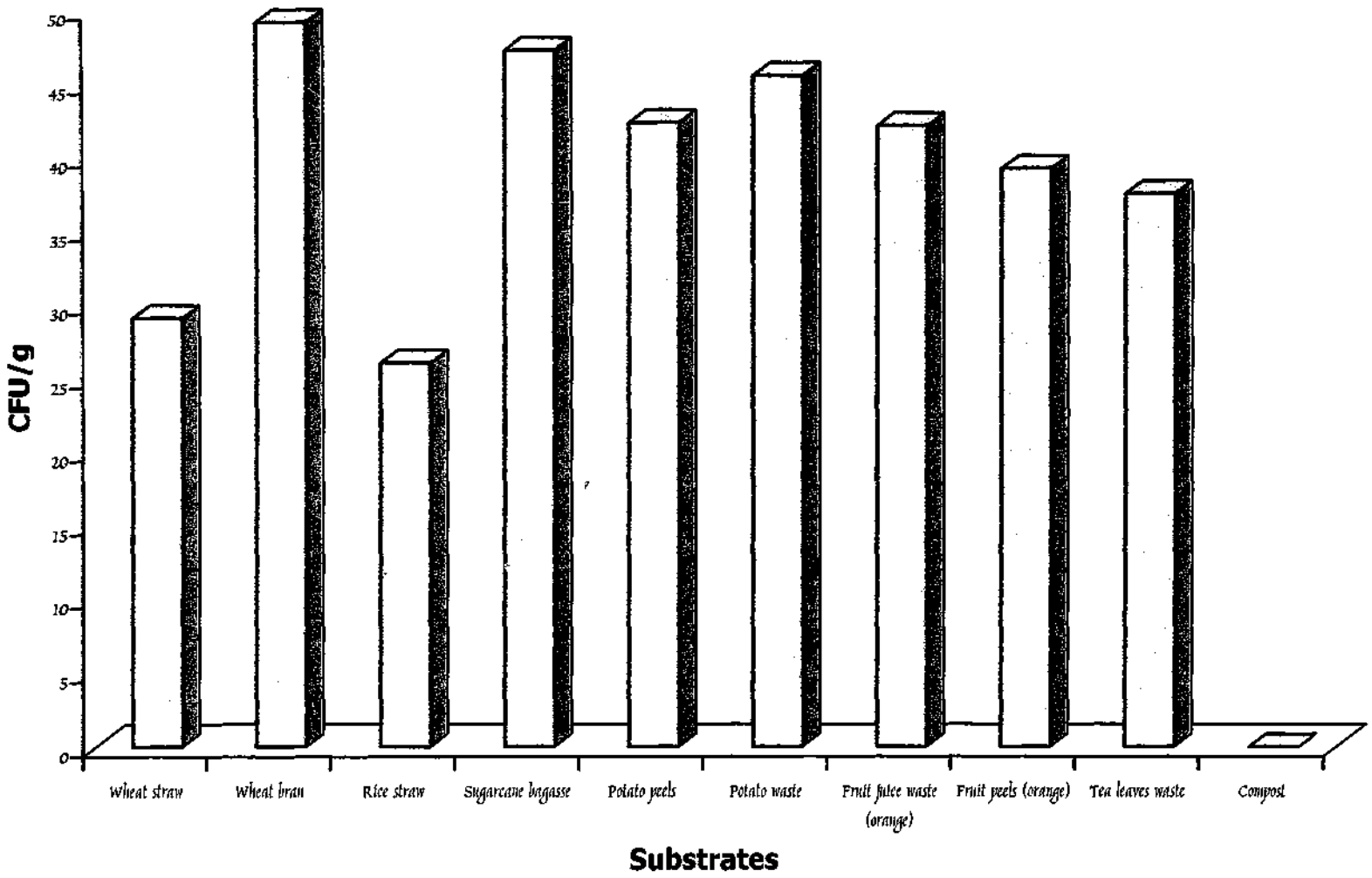


Fig. 16 Effect of different substrates on the growth of *T. harzianum* (TH-1)

Tables 27: Effect of different substrates on the growth of *T. viride* isolate (TV). Data represent mean of 4 replications.

Substrates	CFU/g
Wheat straw	26.8×10^4
Wheat bran	45.9×10^4
Rice straw	29.6×10^4
Sugarcane bagasse	42.8×10^4
Potato peels	36.9×10^4
Boiled potato	36.4×10^4
Fruit juice waste (orange)	41.6×10^4
Fruit peels (orange)	40.9×10^4
Tea leaves waste	40.2×10^4
Compost	-
CD at 5%	0.164

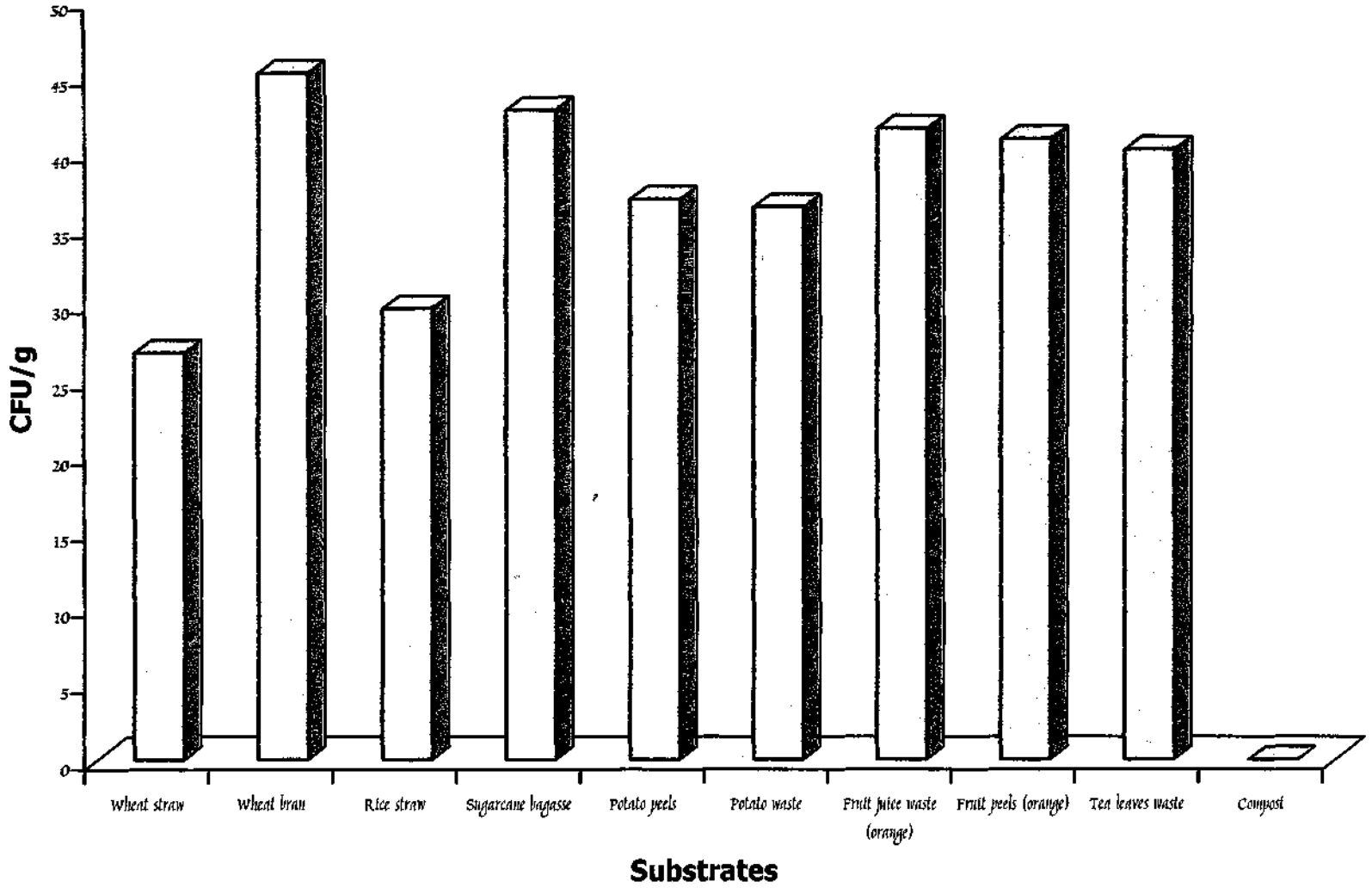


Fig. 17 Effect of different substrates on the growth of *T. viride* (TV)

Chapter – V

SUMMARY

The present investigation was carried out to study the synergistic effect of *R. leguminosarum* bv. *trifolii* isolates and *T. harzianum* on nitrogen fixation in Shaftal (*Trifolium resupinatum* L.) variety Sh-69.

In total four isolates of *T. harzianum* and ten isolates of *R. leguminosarum* bv *trifolii* were isolated from rhizosphere and nodules of Shaftal plants respectively. Isolates of *R. leguminosarum* bv *trifolii* were screened for their efficiency to fix nitrogen under pot culture conditions using sterilized soil. On the basis of observations like number of nodules, their dry weight, leghaemoglobin content, fresh and dry weights of shoot and root, total chlorophyll contents of leaves and nitrogen content of shoot isolate R-1 of *Rhizobium* and isolates TH-1 and TH-4 were found to be efficient followed by R-2, TH-3 and TV respectively.

An *in vitro* experiment was conducted to standardize the cultural conditions (pH, temperature, carbon and nitrogen sources) for the growth of *T. harzianum*. The optimum pH and temperature ranges for growth of *T. harzianum* was found to be 5.5-7.5 and 25-30°C respectively. Regarding nutrient sources glucose as carbon and Ammonium sulphate as nitrogen source was observed to be most efficient to support the maximum growth of *T. harzianum* and *T. viride*. *T. harzianum* isolate (TH-4) was found to have maximum chitinase activity followed by TH-2 isolate.

Seed treatment with culture filtrate of all the isolates of *T. harzianum*

and *T. viride* increased the root length and dry weight of seedlings over uninoculated control. There was 100% emergence of seeds in all the treatments including control but the seeds treated with all the isolates of *T. harzianum and T.viride* showed earlier emergence of seedling as compare to control. *T. harzianum* isolate TH-1 proved better than other three isolates of *T. harzianum* and *T. viride.*

A field experiment was conducted to study the effect of *Trichoderma harzianum* (TH-1), *T. viride* (TV) and *Rhizobium* isolates (R-1) in Shaftal (*Trifolium resupinatum* L.) variety Sh-69. Non-significant increase in green fodder yield was observed in the treatment containing combination of *Rhizobium* and *T. harzianum* of (R-1 + TH-1) as compared to *Rhizobium, T. harzianum* and *T. viride* alone.

Numbers of Substrates (wheat bran, wheat straw, rice straw, sugarcane bagasse, fruit juice waste (orange), fruit peels (orange), tea leaves waste, potato peels, boiled potato and compost) were screened for the mass multiplication of *T. harzianum.* The results indicated that the wheat bran was the most suitable substrate for the growth of *T. harzianum* followed by sugarcane bagasse and then potato waste.

REFERENCES

Ahmad J S and Baker R (1987) Growth of rhizosphere – competent nutrients of *Trichoderma harzianum* on carbon substances. *Can J Microbiol* **34** : 807-14.

Ahmad S J and Baker R (1987) Rhizosphere competence of *Trichoderma harzianum. Pytopathol* 77 : 182-89.

Altomare C, Norwell W A, Bjorkman T and Harman G E (1999) Solubilization of Phosphates and Micronutrients by the Plant – growth promoting and biocontrol fungus *T. harzianum* Rifai 1295-22. *Appl Env Microbiol* : 2926-33.

Amarger N and Lobreau J P (1982) Quantitative study of nodulation competitiveness in *Rhizobium* strains. *Appl Enviorn Microbiol* **44**: 583-88.

Athar M and Doughlas A J (1996) Competitive ability of *Rhizobium melilotii* strains from Pakistan and Nepal for nodulation in three alfa accessions. *J Appl Bot* **70**: 128-33.

Aube C and Gagnon C (1969) Effect of carbon and nitrogen nutrition on growth and sporulation of *Trichoderma viride*. *Can J Microbiol* **15**: 703-706.

Bae Y S, Jang S, Park C, Kim H, Bae Y S, Jang S S, Park C S and Kim H K (1995) *In vitro* and green house evaluation of cucumber growth enhanced by rhizosphere microorganisms. *Korean J Pl Pathol* **11** : 292-97.

Bakker P A, Ran H M, Pieterse L X and Loop L C (2003) Understanding the involvement of *Rhizoctonia* mediated induction of systematic distance in biocontrol of plant disease. *Can J Pl Pathol* **25**: 5-9.

Barhate B G, Bendre N J and Gaikwad R T (1999) Studies on interaction of chickpea cultivars to different strains of *Rhizobium. Legume Research* **22** : 204-206.

Bassam B J and Gresshoff P M (1986) Use of neomycin for preferential relation against *R. trifolii* in symbiosis with white clover (*Trifolium repens*). *Australian J Biol Sci* **39** : 31-36.

Bastos C N (2001) Effect of temperature, pH and nutrition on growth and sporulation of *Trichoderma stromaticum* sp. nov an antagonist of cocoa witches brown pathogens. *Summa phytopathologica* **27**: 73-76.

Batra R, Rewari R B and Jauhri K S (1992) Effect of nitrate application on nodulation and growth of Rajmaah (*Phaseolus vulgaris*) *Env Ecol.* **10**: 70-73.

Batra, L and Ghai S.K (1988) Performance of different forage legume *Rhizobium* symbiotic systems under saline conditions. *Ind J Agri Sci* **58**: 350-53.

Beijerinick M W (1888) Die bacterian der Papillion feen knollchen. *Botany zig* **46** : 726

Bell J V, Stewart A and Rowarth J S (2000) Application method and growing medium affects the response of cucumber seedlings to inoculation with *Trichoderma harzianum. Austral Pl Pathol* **29** : 15-18.

Bergerson F J (1961) Haemoglobin content of legume root nodules. *Biochem Biophys Acta* **50**: 576-78.

Bergerson F J, Tuner G L and Appbeby C A (1973) Studies on physiological role of haemoglobin in soybean root nodules. *Biochemical Biophysical Acta* **292** : 271-82.

Bernaerts M J and Deley J (1963) A biochemical test for crown gall bacteria. *Nature* **197**: 406.

Bhardwaj S D (1970) *Rhizosphere studies in relation to nodulation in two leguminous plants*. Ph. D. thesis. Hindu University, India.

Bhatnagar P S, Tewari V and Singh B D (1988) Host-*Rhizobium* symbiotic interaction in mungbean (*Vigna radiata* L. Wilczek). *Genetica* **42** : 161-68.

Bjorkman T, Blanchard L M and Harman G E (1998) Growth enchancement of shrunken – 2 (Sh 2) Sweet Corn by *Trichoderma harzianum* 1295-22 : Effect of environment stress. *J Am Soc Hort Sci* **123** : 35-40.

Bookerd N, Weber D F and Bezdieek D F (1978) Influence of *R. japonicum* strains and inoculation method on soybean grown in *Rhizobium* populated soil. *Agron J* **70**: 547-49.

Borregaard S (2000) Supresivit (*Trichoderma harzianum*) : "Evaluation of effect trials". *17th Danish Plant Protection Conference Horticulture.* pp 63-65 DJF – Rapport, Havebrug (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 20001005199).

Bostock (2001) Signal interactions in induced resistance to pathogen and insect herbivores. *Eur J Pl pathol* **107**: 103-11.

Brewin N J, Ambrose M J, Downie J A, Casey R and Davies D R (1993) Root nodules, *Rhizobium* and nitrogen fixation. *Pea : genetics molecular–biology–and–biotechnology* 237-290.

Brougham R W (1960) The relationship between the critical leaf area, total chlorophyll content and maximum growth rate of some pasture and crop plants. *Ann Bot* **24**: 463-74.

Broughton W J, Hoh C H Behm, C A and Tung H F (1978) Development of the nitrogen fixing apparatus in the legumes *Centrosema pubescens* Berth; *Vigna uniguiculata* L. *Planta* **139**: 183-92.

Burris R H and Wilson P W (1957) *Methods in Enzymology* **6** : 355.

Butvina O Yu, Tolkachev N Z and Knyanez A V (1997) High competition *Rhizobium* strains: The basis of biopreparation efficiency. *Mikrobiolohicnnyi Zhurnal* **59**: 123-31.

Caba J M, Lluch C, Hervas A and Ligero F (1990) Nitrate metabolism in roots and nodules of *Vicia faba* in response to exogenous nitrate. *Physiologia Plantarum* **79** :531-39.

Carsolio C (1999) Role of the *Trichoderma harzianum* endochitinase gene ech 42 in mycoparasitism. *Appl Env Microbiol* **65** : 929-35.

Cassiolato A M R, Baker R, Melo Is-de and De-Melo I S (1996) Promotion of growth in lettuce plants by *Trichoderma harzianum. Revista de Agricultura Piracicaba* **71** : 55-65.

Chahal V P S and Rewari R B (1975) Leghaemoglobin and bacteriod content in relation to nitrogen fixation. *J Res* (PAU) **XIV** : 386.

Chahal V P S and Sharma P K (1991) Relative behaviour of 4 chickpea cultivars for nitrogen fixation and nitrate utilization. *Crop Improvement* **18**: 46-53.

Chahal V P S, Verma V K and Sharma P K (1982) Genetic markers for drug resistance in *Rhizobium leguminosarum. J Res (PAU)* **19** : 405-07.

Chang Y C and Baker R, Kleifeld O and Chet I (1986) Increased growth of plants in the presence of biological control agent *Trichoderma harzianum. Pl Dis* **70** : 154-48.

Cherif M and Benhamou N (1990) Cytochemical aspects to chitin breakdown during the parasitic action of *Trichoderma spp.* on *Fusarium oxysporum f. sp. radicis-lycopersici. Phytopathol* **80** : 1406-14.

Chet I (1990) Biological control of soil borne plant pathogens with fungal antagonists in combination with soil treatments. In *Biological control of soil Borne Plant Pathogens*. pp : 15-25. D Horn by, Wallingford.

Chopra C L and Subba Rao N S (1967) Mutual relationship among bacteriods, Leghaemoglobin and nitrogen content of Egyptian clover (*Trifolium alexandrium*) and gram (*Cicer arietinum*). *Archiv Microbiol.* **58**: 71-76.

Cliquet S and Scheffer R J (1997) Influence of culture conditions on growth and survival of conidia of *Trichoderma* spp coated on seeds. *Bioc Sci Technol* **7**: 171-81.

Cooper J E (1990) Competition between *Rhizobium* strains for infection and nodulation of legumes. *Agrokemia-es-Talajtan* **39**: 358-62.

Coventry D R, Hirth J R and Reeves T G (1985) Development of population of *Rhizobium trifolii* and nodulation of subterranean clover following the cropping phase in crop pasture rotations in south eastern Australia. *Soil Biol Biochem* **17**: 17-22.

Coventry D R, Hirth J R, Reeves T G and Burnett V F (1985) Growth and nitrogen fixation by subterranean clover in response to inoculation and soil amendment with lime. *Soil Biol Biochem* **17**: 791-96.

Cruz D L, Hidalgo G A, Lora J M, Benitez T, Pintor J A and Liobell A (1992) Isolation and characterization of three chitinases from *Trichoderma harzianum. Eur J Biochem* **206** : 859-67.

Cruz D L, Pintor J A, Benitez T and Liobell A (1995) Purification and characterization of an endo-β 1, 6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. *J Bacteriol* **177** : 6937-45.

Cruz D L, Rey M, Lora J M, Hidalgo G A, Dominguez F, Pintor J A, Liobell A and Benitez T (1993) Carbon source control on β-glucanase chitobiase and chitinase from *Trichoderma harzianum. Arch Microbiol* **159** : 316-22.

Dadson R B and Acquaah G (1984) *Rhizobium japonicum,* nitrogen and phosphorus effect on nodulation, symbiotic N_2 fixation and yield of soybean (*Glycine max* L.) in southern savanna of Ghana. *Field crop Res* **9**: 101-07.

Danielson R M and Davey C B (1973a) The abundance of *Trichoderma* propagules and the distribution of species in forest soils. *Soil Biol Biochem* **5** : 485-94.

Danielson R M and Davey C B (1973b) Non nutritional factors affecting the growth of *Trichoderma* in culture. *Soil Biol Biochem* **5** : 495-504.

Das B C, Roy S K and Bora L C (1997) Mass multiplication of *Trichoderma* species on different media. *J Agri Sci Soc northern east Ind* **10**: 95-100.

Datnoff L E, Nemec S and Pernezny K (1995) Biological control of *Fusarium* crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradicis. Biol control* **5**: 427-31.

Davidson F (1973) The influence of rhizobial strain and soybean variety on nodules weight and N_2–fixation. *Dissertation Abstracts International* **33** : 4077-78.

Davis B J (1964) Disc-gel electrophoresis II : Method and Applications to Human Serum proteins. *Ann NY Acad Sci* **121** : 404-27.

De Meyer, Bigimere G, Elad Y and Holte M (1998) Induced Systemic resistance in *Trichoderma harzianum* T - 39 biocontrol of *Botrytis cinerea. Eur J Pl Pathol* **104**: 276-88.

Deka K C and Kakati W N (1996) Effect of *Rhizobium* strains, methods of inoculation and levels of phosphorus on mungbean (*Vigna radiata* (L.) Wilezek). *Legume Research* **19** : 133-39.

Dennis C and Webster J (1971a) Antagonistic properties of specific groups of *Trichoderma*-I. Production of volatile antibiotics. *Trans Br Mycol Soc* **57** : 25-39.

Dennis C and Webster J (1971b) Antagonistic properties of specific groups of *Trichoderma*-II. Production of volatile antibiotics. *Trans Br Mycol Soc* **57** : 363-69.

Dubach M and Ruselle M P (1994) Forage legume roots and nodules and their role in nitrogen transfer. *Agron J* **86**: 259-66.

Duodu S, Bhuvaneswari T V, Stokkermans T U W and Peters N K (1999) A positive role for rhizobitoxine in *Rhizobium*-legume symbiosis. *Molecular Plant Microbe Interaction* **12** : 1082-89.

Elad Y (1994) Biological control of grape grey mould by *Trichoderma harzianum. Crop protec* **13**: 35-38.

Elad Y, Chet I and Henis Y (1981) A selective medium for improving quantitative isolation of *Trichoderma spp.* from soil. *Phytoparasitica* **9** : 59-66.

Elad Y, Chet I and Henis Y (1982) Degradation of plant pathogenic fungi by *Trichoderma harzianum. Can J Microbiol* **28** : 719-25.

Ezzi M L and Lynch J M (2002) Cyanide catabolizing enzymes in *Trichoderma* spp. *Enzymol Microbiol Technol* **31**: 1042-47.

Fabiano E and Arias A (1991) Competition between native isolates of *R. leguminosarum* bv. *trifolii* and two commercial inoculant strains for nodulation of Clover. *Pl Soil* **137**: 293-96.

Feng Y, Pan C M, Wang D Q and Wei C F (1997) Isolation of nodule bacteria from *Pisum sativum* and the application of nitragin from the isolates. *J Trop subtrop Bot* **5**: 47-53.

Friedericks J B, Hagedorn C and Vanscoyac S W (1990) Isolation of *Rhizobium leguminosarum* bv. *trifolii* strain from Ethiopian soils and symbiotic effectiveness on African annual clover species. *Appl Env Microbiol* **56**: 1087-92.

Fruzynska J D and Manka M (1994) Biotic series method for evaluation of soil fungi effect on plant pathogenic fungi II. Effect of medium pH, medium amendments and temperature on individual biotic effect value. *Phytopathologia – polonica* **19**: 131-36.

Furlani J, Eves J N, Luciano J B, Jose A M and Hullo G F (1996) Correlation between chlorophyll content and nitrogen levels applied on bean leaves. *Bragantia* **55**: 171-75.

Gandhikumar N, Raguchander T and Prabakar K (2001) Mass multiplication of biocontrol agent : a cost effective approach. *Ann Pl Protec Sci* **9**: 140-42.

Gao W M and Yang S S (1995) A *Rhizobium* strain that nodulates and fixes nitrogen in association with alfalfa and soybean plants. *Microbiology Reading* **141** : 1957-62.

Ghisalberti E L and Sivasithamparam K (1991) Antifungal antibiotics produced by *Trichoderma* spp. *Soil Biol Biochem* **11** : 1011-20.

Gindrat D and Ricard J L (1976) Counting techniques for *Trichoderma viride* conidia dispersed in barley flour inoculants *Pl Dis Reptr* **60** : 321-25.

Gok M and Martin P (1993) Effect of inoculation with various *Rhizobium* species on molecular nitrogen fixation by Soybean, Clover and vetch plants. *Doga Turk Tarim ve ormancilik Dergisis*, 17 : 753-67.

Gonzalez S CH, Rodriguez L L, Arjona C, Puertas A and Fojseca M (1999) Effect of *Trichoderma harzianum* application on quantitative composition of bacteria, fungi and actinomycetes in the solanaceae rhizosphere and its influence on vegetative growth. *Investigacion Agraria, Produccion Y protecd on vegetables* **14** : 297-306.

Gourret J P and Fernandes Arias H (1979) *Can J Microbiol* **20**: 1169-74.

Graham P H and Vance C P (2000) Nitrogen fixation in perspective : an overview of research and extension needs. Special issue, *Applied Technologies in biological Nitrogen fixation field crop research* **65** : 2-3.

Gregr V (1990) Nutrient intake in winged bean and cowpea in relation to inoculation of the seed. *Agricultura-Tropica-et-Subtropica* **23** : 39-46. (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 910741508).

Grondona I, Hermosa R Tejada M, Gomis M D, Mateos P F, Bridge P D, Monte E and Garcia-acha I (1997) Physiological and biochemical characterization of *Trichoderma harzianum*, a biological control agent against soil borne fungal pathogens. *Appl Env Microbiol* : 3189-98.

Gupta S C and Namedo S L (1996) Effect of *Rhizobium* strains on symbiotic traits and grain yield of chickpea. *Indian Journal of Pulses Res* **9** : 94-95.

Hahn J (1966) Congo red reaction in bacteria and its usefulness in identification of rhizobia. *Can J Microbiol* 19 : 125.

Haran S, Schickler H and Chet I (1996) Molecular mechanisms of the lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum. Microbiol* **142** : 2321-31.

Hari K and Somasekhar N (1998) Utilization of sugarcane wastes for the mass multiplication of fungal biocontrol agents. *Cooperative Sugar* **29**: 637-38.

Harman G E (2000) Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Pl Dis* **84**: 377-93.

Harman G E (2001) Mode of action and application *Proc in intl sym on Biological control of plant disease for the new century* pp 71-84.

Harman G E, Chet I and Baker R (1981) Factors affecting *Trichoderma hamatum* applied to seeds as a biocontrol agent. *Phytopathol* 71 : 569-72.

Harman G E, Howell C R, Viterboa, Chet I and Lorito M (2004) *Trichoderma* species opportunities, a virulent plant symbionts. *Nature Rev* 43-55.

Harman G E, Taylor A G and Stasz T E (1989) Combining effective strains of *Trichoderma harzianum* and solid matrix priming to improve biological seed treatments. *Pl Dis* **73** : 613-37.

Heemert K Van, Veenstra H, Jorgensen H and Heemert K Van (2000) TRI 002 and TRI 003 new biological plant growth stimulators. *17th Danish Plant Protection Conference, Horticulture*. pp 17-80. DJF – Rapport, Havebrug.

Hellriegel H and Wilfarth H (1888) Beilageheft Zu der ztschr ver. Ruben zuckker industrie Deut Chen Reichns. 234.

Herridge D F, Rupela O P, Serroj R, Beck D P, Muehlbaver F J and Kaiser W J (1994) Screening techniques and improved biological nitrogen fixation in cool season feed legumes. *Proc 2nd Int Food Legume Research Conference on pea, lentil bababeans, chickpea and grass pea*. pp 472-92. Cairo Egypt.

Hervas A, Caba J M, Ligero F and Lluch C (1991) Effect of combined nitrogen supply and nodulation on nitrate reductase activity and growth of pea plants. *J Pl Nut* **14**: 1319-30.

Hervas A, Ligero F and Lluch C (1991). Nitrate reduction in pea plants: Effect of nitrate application and *Rhizobium* strains. *Soil Biol. Biochem.* **23**: 695-99.

Huang, C Y and Chiying S C (1982) Effect of NH_4NO_3 on the activity of N_2 fixing and assimilatory enzymes in the nodule. *Proc Natl Sci Coun Rep China* Part – B. Basic. **6** : 326-31.

Hyakumachi M (1994) Plant-growth promoting fungi from turfgrass rhizosphere with potential for disease suppression. *Soil Microorganisms* **44** : 53-68.

Ibekwe A M, Angle J S, Cheney R L and Berkum P V (1997) Differentiation of Clover *Rhizobium* isolated from bacteriod amended soils with varying pH. *Soil Sci Soc Am J* 61: 1679-85.

Jackson A M, Whipps J M and Lynch J M (1991) Effect of temperature, pH and water potential on growth of four fungi with disease biocontrol potential. *World J Microbial Biotechnol* 7 : 494-01.

Jackson A M, Whipps J M, Lynch J M and Bazin M S (1991) Effects of three carbon and nitrogen sources on spore germination, production of biomass and antifungal metabolites by species of *Trichoderma* and *Gliodadium virens* antgonisitic to *Sclerotium cepivorum*. *Bio Sci Technol* **1** : 43-51.

Jaworski E G (1971) Nitrate reductase assay in intact plant tissue. *Biochemical Biophysical Research Communication* **43** : 1274-78.

Jayaraj J and Ramabadran R (1996) Evaluation of certain organic substrates and adjuvants for the mass multiplication of *Trichoderma harzianum* Rifai. *J Biol control* **10**: 129-31.

Jayaraj J and Ramabadran R (1999) *Rhizobium-Trichoderma* interaction *in vitro* and *in vivo. Ind Phytopath* **52** : 190-92.

Jimbo, T (1930) On the Serological classification of the root nodule bacteria of leguminous plants. *Bot Magazine* **44**: 158-68.

Johnson A W B and Beringer J E (1976) Mixed inoculations with effective and ineffective strains of *Rhizobium leguminosrum. J Appl Bacteriol* **40**: 375-80.

Johnson H S and Homes D J (1973) Comparison of N_2 fixation and estimate in soyabean by nodule weight, Leghaemoglobin content and acetylene reduction. *Can J Microbiol* **19**: 1165-68.

Josephson K L and Pepper I L (1984) Competitiveness and effectiveness of strains of *Rhizobium phaseolus* isolated from sonoran desert. *Soil Biol Biochem* **16** : 651-56.

Josey, D P, Beynon, J C, Johsten A W B and Beringer J E (1979) Strain indentification in *Rhizobium* using intrinsic antibiotic resistance. *J Appl Bacteriol* **46** : 343-50.

Kastov O (1996) Growth and survival of *Rhizobium* and *Bradyrhizobium* stains on composted paper sludge. *Pochvoznanie Agrokhimiia Y Ekologiya* **31** : 19-20 (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 931002082).

Kehri H K and Chandra S (1991) Antagonism of *Trichoderma viride* to *Macrophomina phaseolina* and its application in the control of dry root-rot of mung. *Ind Phytopath* **40** : 60-63.

Keilen D K and Wang Y C (1945) Haemoglobin in the root nodules of leguminous plants. *Nature* **155** : 223

Khurana A L and Dudeja S S (1981) Response of chickpea to *Rhizobium* and nitrogen on nitrogen fixation and grain yield. *Pulses Crop Newsletters* **1** : 105.

Kishinerisky B D, Leshans Y, Friedman Y and Krivatz G (1992) Yield and nitrogen winter of berseem clover as a potential winter forage crop under semi arid conditions. *Arid Soil Research and Rehabiliation.* **6**: 261-71.

Kleifield O and Chet I (1992) *Trichoderma harzianum* interaction with plants and effect on growth response. *Pl Soil* **144** : 267-72.

Kubo H (1939) Uber Hamoprotein aus den Wurzel Knalden von leguminosen. *Acta Phytochem* **11** : 195-201.

Kuc J (2001) Concepts and direction of induced systematic resistance in plants and its application. *Eur J Pl Pathol* **107**: 7-12.

Kumar A and Gupta J P (1999) Variation in enzyme activity of Tebuconazole tolerant biotypes of *Trichoderma viride*. *Ind Phytopathol* **52**: 263-66.

Kumar T K D, Rao A R M, Reddy S M, Srivastava H P, Purodit D K and Reddy S R (1997) Screening of *Rhizobium* isolates for symbiotic efficiency in pot culture. Microbiol Biotechnology. "*Professor K S Bligrami Commemoration Volume*". pp 70-74.

Larenas C and Montealegre J R (1996) Effect of storage temperature and nutrient amount on the viability of *Trichoderma harzianum* pellets. *Fitopathologia* **31**: 66-69.

Lee J S, Cho K J, Nm N H and Khang U G (1990) Influence of gypsum and micro-elements fertilization in groundnut with inoculation of *Rhizobium* spp. *Soil and Fertlizers* **32** : 47-51.

Limon M C, Pintor J A and Benftez T (1999) Increased antifungal activity of *Trichoderma harzianum transformants* that overexpress a 33-kDa Chitinase. *Phytopathol* **89**: 254-61.

Lo C T, Nelson E B, Hayer C K and Harman G E (1999) Ecological Studies of transformed *Trichoderma harzianum* strain 1295-22 in the rhizosphere and on the phyllophane of creeping bentgrass. *Phytopathol* **88**: 129-36.

Lowry H O, Rose brough N J, Far A L and Randal R L (1951) Protein measurement with folin Phenol reagent. *J Biol Chem* **193**: 263-75.

Mahadkar U V and Saraf C S (1988) Nitrate reductase in relation of inoculation and nitrogen nutrition of green gram. *Journal of Maharashtra Agricultural University* **13** : 291-93.

Malathrakis N E, Kritsotaki O and Verhoeff K (1992) Effect of substrate, temperature and time of application on the effectiveness of three antagonistic fungi against *Botrytis cinerea*. Recent advances in *Botrytis* research. *Proc 10th Intl Botrytis Symp* pp 187-91.

Maldal A B and Ray R (1999) Effect of *Rhizobium* inoculation and nitrogenous fertilizer on the performance of moong. *Journal of Interacademicia* **3** : 259-62 (Original not seen. Abstr in CAB Abstracts, CD-ROM, An 20000706439).

Martin J P (1950) Use of acid, rose bengal and streptomycin in the plate method for estimating soil fungi. *Soil Sci* **69** : 215-32.

Mcloughlin T J and Dunican L K (1985) An ecological study of marked *R. trifolii* strains on the host plant *Trifolium repens* var. Huai in an acidic peat and a neutral mineral *J Appl Bacteriol* **50**: 65-72.

Mcloughlin T J, Bordeleav L M and Dunican L K (1984) Competition studies with *Rhizobium trifolii* in a field experiment. *J Appl Bacteriol* **56**: 131-35.

Metchell D A and Wilson C R (2001). The process of antagonism of *Sclerotium cepivorum* in white roots by *Trichoderma koningii. Pl Pathol* **50**: 249-57

Michalikova A (1995) The influence of a biopreparation. Trichonitrin on the growth and development of winter wheat during the seedling stage. *Acta Fytotechnica* **50** : 125-29.

Monga D (2001) Effect of carbon and nitrogen sources on spore germination, biomass production and antifungal metabolites by species of *Trichoderma* and *Gliocladium. Ind J phytopathol* **54**: 435-37.

Nakkeeran S, Sankar P and Jeyarajan R (1997) Standardization of storage conditions to increase the shelf life of *Trichoderma* formulations. *J Mycol Pl Pathol* **27**: 60-63.

Naseby D C, Pascual J A and Lynch J M (2000) Effect of biocontrol strains of *Trichoderma* on plant growth, *Pythium ultimum* population, soil microbial communities and soil enzyme activities. *J Appl Microbial* **88** : 161-69.

Nelson (1987) Variation in *Rhizobium leguminosarum* response in short term application on ammonium nitrate to nodulate *P. sativum. Pl Soil* **98**:275-81.

Nemec S, Datnoff L E and Strandberg J (1996) Efficiency of biocontrol agents in plant mixes to colonize plant roots and control root disease of vegetable and citrus. *Crop Prot* **15**: 735-42.

Ohtakara A (1988) Chitinase and βN-Acetylhexosaminidase from *Pycnoporus cinnabarainus. Method Enzymol* **161**: 467-68.

Oostendorp M, Kunz W, Detrich B and Staub T. (2001) Induced disease resistance in plants by chemicals. *Eur J Pl Pathol* **107**: 19-28.

Ouazzani- Touhami A, Mouria A, Douira A, Benkirane R, Miaikia A, E L and Yachioui M (1997) *In vitro* effect of pH and temperature on the ability of *Trichoderma* spp to reduce the growth of *Pyricularia oryzae. Al-Awamia* **96**: 19-24.

Papavizas G C (1985) *Trichoderma* and *Gliocladium* biology, ecology and potential for biocontrol. *Annu Rev Phyopathol* **23**: 23-54.

Parco S Z, Dilworth M J and Glenn A R (1994) Motility and the distribution of introduced root nodule bacteria on the root system of legumes. *Soil Biol Biochem* **26**: 297-300.

Patra D K and Bhattacharya P (1997) Influence of root nodule bacterium on nitrogen fixation and yield of mungbean. *J Mycopathol Res* **35** : 47-49.

Patra D K and Bhattacharya P (1998) Response of cowpea rhizobia on nodulation and yield of mungbean (*Vigna radiata* (L.) Wilczek). *J. Mycopathol Res* **36** : 17-23.

Pieterse C M (2001) Rhizobacteria mediated induced systemic resistance triggering signaling and expression. *Eur J Pl Pathol* **107** : 51-61.

Poi S C and Kabi M C (1982) Response of winged bean (*Psophocarpus tetragonobus*) to rhizobial inoculation and its prospects in West Bengal. *Tropical Grain Legume Bulletin* **24** : 24-25.

Posypanov G S, Shilovskaya K A, Chernova V I and Chernov B A (1990) Compatibility of red clover cultivars and *Rhizobium* strains. *Izvestiya Timirya-zevskoi-sel Skokhozyaistvenuoi Akademir* **4** : 92-98.

Prabhakaran J and Ramaswamy K (1990) Effect of seed dressing chemicals on the survival of *Rhizobium* sp. and growth, nodulation and grain yield of green gram and blackgram. *Madras Agricultural Journal* 77 : 285-90.

Prakash M G, Gopal K V, Anandraj M and Sarma Y R (1999) Evaluation of substrates for mass multiplication of fungal biocontrol agents *Trichoderma harzianum* and *T. virens. Journal of Spices and Aromatic Crops* **8**: 207-10.

Prasad R D and Rangeshwaran R (2000) An improved medium for mass production of the biocontrol fungus *Trichoderma harzianum. J Myc Pl Path* **30** : 233-25.

Pugh R, Mytton L R and Minchin F R (1995) The effect of logging on nitrogen fixation and nodule morphology in soil grown white clover (*Trifolium repens* L.). *J Exp Bot Oxford.* **46**: 285-90.

Pushnik J C, Miller G W and Marwaring J H (1984) The role of iron in higher plant chlorophyll biosynthesis, maintenance and chloroplast biogenesis. *J Pl Nut* **7**: 733-58.

Rai R (1992) Effect of nitrogen levels and *Rhizobium* strains on symbiotic N_2 fixation and grain yield of *Phaseolus vulgaris* L. genotypes in normal and saline-sodic soils. *Biology and Fertility of Soil* **14** : 293-99.

Rajput A L and Singh T P (1996) Response of nitrogen and phosphorus with and without *Rhizobium* inoculation and fodder production of cowpea (*Vigna unguiculata*). *Ind J Agron* **41** : 91-94.

Rani B and Kodandaramaiah D (1997) Response of soybean (*Glycine max*) to *Rhizobium* inoculation under varying nitrogen levels. *Ind J Agron* **42** : 135-37.

Rasal P H, Veer D M and Patil P L (1989) A comparative study on the performance of *Rhizobium* inoculants obtained from different sources. *Madras Agricultural Journal* **76** : 342-44.

Renwick A and Jones D G (1985) A comparison of fluorescent ELISA and antibiotic resistance identification techniques for use in ecological experiments with *Rhizobium trifolii*. *J Appl Bacteriol* **58**: 199-206.

Rollan M, Monaco C and Nico A (1999) Temperature effect on *in vitro* interactions between *Trichoderma* spp. and *Sclerotinia sclerotiorum, S. minor* and *Sclerotium rolfsii. Investigacion Agraria Produccion Y Proteccion Vegetables* **14** : 3348.

Roughly R J, Blowes W M and Herridge D F (1976) Nodulation of *Trifolium subterraneum* by introduced rhizobia in competition with neutralized strain. *Soil Biol Biochem* **8**: 403-07.

Saha D K and Pan S (1998) Factors effecting survival potential of *Gliocladium vircns* in soil. *Ind Phytopathol* **51** : 51-56.

Sairam P K, Tomar P S, Harika A S and Ganguly T K (1989) Effect of phosphorus levels and inoculation with *Rhizobium* on nodulation leghaemoglobin content and nitrogen uptake in fodder cowpea. *Legume Research* **12** : 27-80.

Sangakkara U R (1993) Relationship between soil moisture, growth yields and nitrogen fixation in selected grain legumes. *Acta Agronomica Hungarica* **42** : 162-69 (Original not seen. Abstr in CAB Abstracts, CD – ROM, AN 950711492).

Sarode S V, Gupta V R and Asalmol M N (1998) Suitability of carriers and shelf life of *Trichoderma harzianum*. *Ind J Pl Prot* **26** : 188-89.

Sawhney V, Amarjit and Singh R (1985). Effect of applied nitrate and growth and N_2 fixation in (*Cicer arietinum* L.) *Pl Soil* **86** : 233-40.

Scholander P P (1960) Oxygen Transport thro' haemoglobin solution. *Sci* **131**: 585-90.

Schwinghamer E A and Dudman W F (1973) Evaluation of spectinomycin resistance as a marker for ecological studies with *Rhizobium* spp. *J Appl Bacteriol* **36** : 263-72.

Shamarao J, Siddaramaiah A L, Narayana Swamy H, Jahagirdar S (1998) Screening of substrates for mass multiplication of *Trichoderma viride*. *Karnatka J Agri Sci* **11** : 233-36.

Sharma, P.K and Chahal V.P.S (1983). Effect of Molybdenum and boron on nodulation and dry matter of Lentil. *J Res PAU.* **20** : 563-71.

Shimakara K and Takiguchi Y (1988) Preparation of crustacean Chitin. *Method Enzymol* **162** : 423.

Shivana M B, Meera M S and Hyakumachi M (1994) Sterile fungi from Zoysiagrass rhizosphere as plant growth promoters in spring wheat. *Can J Microbiol* **40** : 637-44.

Shivana M B, Meera M S, Kageyama K and Hyakumachi M (1996) Growth, promotion ability of Zoysiagrass rhizosphere fungi in consecutive plantings of wheat and soyabean. *Mycosci* **37** : 163-68.

Shukla S K and Dixit R S (1996) Nutrient and plant population management in summer greengram (*Phaseolus radiatus*). *Current Research* **19**: 101-102.

Siddiqui I A, Ehteshamul H S and Ghaffaro A (1998) Effect of rhizobia and fungal antagonists in the control of root infecting fungi on sunflower and chickpea. *Pak J Bot* **30**: 279-86. (Original not seen. (CAB Abstracts, Entry No. 19991005235, 1998).

Sindhu S S and Dadarwal K R (2000) Competition for nodulation among rhizobia in legume – *Rhizobium* symbiosis. *Ind J Microbiol* **40** : 211-46.

Singh, V, Wandwal A.S, Bharti S and Kundu B.S (1994) Induced nodule senescence and nitrogen metabolism at various growth rates in Chickpea (*Cicer arietinum* L.). *Ind J Pl Physiol* **37**: 152-56.

Skrdleta A, Gaudinova M, Nencova M and Hyndrakovea A (1980) Symbiotic dinitrogen fixation as affected by short term application of nitrate to nodulate *Pisum sativum* L. *Folia Microbiol* **25**: 155-61.

Skrdleta V and Karimora J (1961) Competition between two somatic serotypes of *R. japonicum* used as double strain inocula in varying proportions. *Arch Microbiol* **66**: 25-28.

Skrobakova E (1995) Effect of growth regulators on yield formation in garden pea. *Agrochemica Bratislava* **35** : 61-62.

Smith J D (1949) The concentration and distribution of haemoglobin in root nodules of leguminous plants. *Biochem J* **44**: 585-91.

Smith N R and Dowson U T (1944) The bacteriostatic action of rose Bengal in media used for plate counts of soil fungi. *Soil Sci* **58** : 467-71.

Terry N (1983) Limiting factors in photosynthesis. IV. Iron stress mediated changes in light harvesting and electron transport capacity and its effect on photosynthesis, *In vivo. Pl Physiol* **71**: 855-60.

Thies J E, Bohlool B B and Singleton P W (1992) Environmental effects on competition for nodule occupancy between introduced and indigenous rhizobia and among introduced strain. *Can J Microbiol* **38** : 493-500.

Thrane C J, Tronsmo A and Jensen D F (1997) Endo-1-3-β-glucanase and cellulase from *Trichoderma harzianum* purification, partial characterization and biological activity against plant pathogenic *Pythium* spp. *Eur J Pl Pathol* **103**: 331-44.

Trouchet, G (1972) Comptes, Rend. *Acad. Sci* Paris. **274**: 1290.

Tsao P H (1970) Selective media for isolation of pathogenic fungi. *Annl Rev Phytopath* **8** : 157-86.

Valdivia B, Dughri M H and Bottomley P J (1988) Antigenic and symbiotic characterization of indigenous *Rhizobium leguminosarum* bv. *trifolii* recovered form root nodules of *Trifolium pratensi* L. sown in to subterranean clover pasture soils. *Soil. Biol. Biochem* **20**: 267-74.

Vincent J M (1963) Taxonomically significantly group antigens in rhizobia. *J Gen Microbiol* **63**: 379-82.

Vincent J M (1970) *A manual for practical study of the root nodule bacteria.* Ist ed Blackwell Scientific Publications, Oxford Fdinburgh.

Viratanen A I (1947) The biology and chemistry of nitrogen fixation by legume bacteria. *Biol Rev* **22**: 239-69.

Whipps J M (2001) Microbiol interactions and biocontrol in the rhizosphere. *J Exp Biol* **52**: 487-511.

Wilson D O and Reisenauer (1963) Determination of leghaemoglobin in

Windham M T, Elad Y and Baker R (1986) A mechanism for increased plant growth induced by *Trichoderma* spp. *Phypathol* **76** : 518-21.

Witham P H, Baidhyes D F and Delvin R M (1971) Chlorophyll absorption of spectrum and quantitative determinations. *Experiments in plant physiology* Published by Von Nostrand Reinhold Company, New York.

Wu W S (1982) Seed treatment by applying *Trichoderma* spp. to increase emergence of soybeans. *Seed Sci Technol* **10** : 557-63.

Xu D Q, Shen Y G, Wang S J and Zand X W (1989) Study of relationship between photosynthesis and N fixation in the symbiotic system of soybean and rhizobia. *Acta Botanica Sinica* **31** : 103-09.

Yaman M (1995) Effect of *Rhizobium japonicum* strains on nodulation and amount of nitrogen fixation of M1 and M2 generations of irradiated soyabean seeds. *Andolu* **5** : 1-10. (Original not seen. Abstr in CAB Abstracts, CD-ROM, AN 960700142).

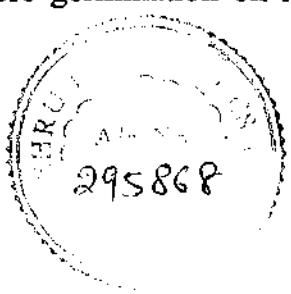
Yedida L, Benhamou N and Chet I (1999) Induction of defence responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum. Appl Env Microbiol* **65** : 1061-70.

Yedida L, Benhamou N, Kapulnik Y and Chet I (2000) Induction and accumulation of PR activity during early stages of root colonization by mycoparasite *Trichoderma harzianum* strain T – 203. *Pl Physiol Biochem* **38** : 863-73.

Yedida L, Benhamou N, Kapulnik Y and Chet I (2004) Concomitant induction of systemic–resistance to *Pseudomonas syringae* pv. *Lachymanas* in cucumber by *Trichoderma asperellum* (T-203) and accumulation of Phytoellexins. *Appl Env Microbiol* **161**: 1863-72.

Yocum C S (1964) Recent studies on symbiotic nitrogen fixation. *Sci.* **146**: 432.

Yoder D L and Lockwood J L (1973) Fungal spore germination on natural and sterile soil. *J Gen Microbiol* **74** : 107-17.



VITA

Name	:	Pooja Khanna
Father's Name	:	Mr. Puran Chand Khanna
Mother's Name	:	Mrs. Chanchal Khanna
Nationality	:	Indian
Date of Birth	:	31st January, 1979
Permanent Address	:	H.No. 199, Dayal Nagar Ghumar Mandi Ludhiana - 141 001

EDUCATIONAL QUALIFICATIONS

Bachelor's degree	:	B.Sc. (Medical)
University	:	Panjab University, Chandigarh
Year of award	:	1999
%age	:	79
Master's degree	:	M.Sc. (Microbiology)
University	:	Punjab Agricultural University, Ludhiana,
Year of award	:	2001
OCPA	:	7.96/10.00

Ph.D.

OCPA	:	7.52/10.00
Title of Master's Thesis	:	Effect of *Rhizobium* and *Trichoderma harzianum* on nitrogen fixation and growth characteristics of moongbean (*Vigna radiata* L.)
Awards/Distinctions/ Fellowship/Scholarship	:	• Merit Certificate Holder in B.Sc. • Fellowship Holder in M.Sc. Ist year • NET qualified (ICAR)