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Study the *In vitro* and *In vivo* both bio-efficacy of *Trichoderma* strains against *Fusarium oxysporum* f. spp. *Lentis* and *Sclerotium rolfsii*

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Abstract
The disease appears in the field in patches at both seedling and adult stages. Seedling wilt is characterized by sudden drooping, followed by drying of leaves and seedling death. The roots appear healthy, with reduced proliferation and nodulation and usually no internal discoloration of the vascular system. Significant highest radial mycelium growth rate was observed in *T. viride* (90%) and followed by *T. harzianum-4* (89%), *T. harzianum-5* (87%). The mycelial growth of *T. harzianum-5* and *Sclerotium rolfsii* in dual culture were 66.2 mm and 24.2 mm followed by *T. viride* 65.1mm and 26.3 mm respectively after 168 hrs of incubation. The mycelial growth of *T. viride* and *Fusarium oxysporum f. sp. lentis* in dual culture were 75.3 mm and 15.7 mm followed by *T. harzianum-5* 70.1 mm and 20.6 mm respectively after 168 hrs of incubation. Significant higher germination per cent of lentil seed was recorded in *T. viride* (74.5%) followed *by T. harzianum-5* (72.5%) as compared to control (56.5%) in field condition. *Trichoderma* strains significantly reduced collar rot incidence was recorded in *T. viride* (13%) followed by *T. harzianum-5* (14.25%) as compared to control (32.5%). *Trichoderma* strains significantly reduced wilt incidence was recorded in *T. viride* (14.25%) followed by *T. harzianum-5* (15.30%) as compared to control (35.25%).

Keywords: Significant, *Trichoderma* strains, healthy, lentil and discoloration

1. Introduction
Lentil production in India has always been important as it is one of the most important rabi crops in the country. Lentil (*Lens culinaris Medik.*) is an important pulse crop and the second major source of protein (25%) after soybean in human and animal diet (Rahman *et al*., 2010) [7]. Lentil is important source of energy (353 kcal), protein (25%), carbohydrate (63%), sugar (2%), fat (1%), vitamin and antioxidant compounds. The morphological and cultural characters of *Fusarium* are similar, among isolates, which cause wilt of pulses, the fungus is host specific so called as formae speciales. Each formae speciales produce different symptoms. *Fusarium oxysporum* Schlecht and Emnd Snyd. & Hans. f.sp. *ciceri* (Padwick) Snyd. & Hans. Affects chickpea, *Fusarium oxysporum* f. sp. udum Butler affects pigeon pea, *Fusarium oxysporum* Schl. f. sp. *pisi* (van Hall) Snyd. & Hans. affects pea and *Fusarium oxysporum* f.sp. *lentis* affect lentil Sinha *et al*. (2018) [9]. Collar rot disease caused by *S. rolfsii* on lentil crop is very important as the polyphagus pathogenic fungus causes substantial losses in quality and productivity of yield. *S. rolfsii Sacco* is a non specialized soil borne fungal pathogen of worldwide importance and has a host range of over 500 species (Punja and Grogan, 1988) [6].

2. Material and Methods
2.1. *In vivo* and *In vitro* test
2.1.1. Equipments and apparatus
The equipments and apparatus which have been used in the study are given below:-Laminar air flow, BOD incubator, Refrigerator, Autoclave, Glassware, Microscope, Hot air oven, Electronic balance, Forceps, Inoculation Needle, Cork borer, Blade etc.

2.1.2. Cleaning and sterilization of equipments
Corning make glassware were used during the period of investigation. All the glassware were cleaned with chronic acid, followed by thorough washing with detergent powder and then rinsing tap water before use. The sterilization of media was done at 15lbs, pressure for 20 min. Petri plates were sterilized in hot air sterilizer at 180 °C for 2 hrs. The plastic Petri plates used in bio control study, were sterilized by alcohol. The isolation chamber was sterilized by alcohol, followed by ultraviolet exposure for 20 min. The other equipments used in isolation chamber like forceps, inoculation needle, cork borer, blade, etc. were sterilized by dipping them in alcohol, followed by heating on flame.

2.1.3. Morphology
Temporary slides were prepared from pure culture. Calibrated ocular was used for measurement of hyphae, conidia and conidiophores. The length and width of conidia and conidiophores along with width of hyphae were measured with the help of calibrated ocular micrometer.

2.1.4. Unit of measurement
The unit of measurement was µm (1µm = 1/1000mm = 10^{-6} m).

2.1.5. Micrometers Ocular micrometer
The scale contained 100 divisions in grade 10, 20, 30, up to 100. The value of one division of the scale varied from micrometer to micrometer. Therefore, calibration of ocular micrometer was made with the help of stage micrometer to record the value of one division of the ocular.

2.1.6. Stage micrometer
It consisted of 1mm scale divided into 100 equal divisions. Therefore, 1 divisions = 0.01mm = 10µm (1mm = 1000µm).

2.1.7. Calibration
For calibration of ocular, it was first placed inside the eye piece of 10 X and stage micrometer was placed on the stage of the microscope. The stage micrometer was placed under focus and ocular divisions were coincided with divisions of stage micrometer and calculation was made by the following procedure.

2.1.7.1. Microscope No
Eye piece: 10x
Objective: 10x
Since 100 divisions of stage micrometer = 1mm

2.2. Methods of inoculation
For inoculating different solid media in Petri- plates, 7 days old culture grown on potato dextrose agar medium was used. The small size of the inoculum was cut and placed at the centre of the plate in an inverted position, so that it came in direct contact with the surface of the medium. For inoculating different liquid media in 100 ml Erlenmeyer flasks containing 25 ml broth medium, one disc of 5 mm diameter of fungal mycelium was allowed to float on the medium.

2.3. Incubation
The inoculated Petri – plates and flasks were incubated at 28 ± 1 °c in B.O.D. incubator for required period.

2.4. Measurement of radial growth of colony
Radial growth of the regular colonies was measured in two directions at right angles with help of a linear scale. In case of irregular colonies, measurements were recorded at the broadest and narrowest diameter and average of two different directions was taken as growth. In all the cases radial growth was recorded after 168 hrs of incubation. In case of poisoned food techniques, it was recorded after 120 and 168 hrs of incubation.

2.5. Preparation of culture media
The various culture media were prepared according to the standard formulae given by Khare *et al.*, (1974) [3]. The constituents and method of preparation of various solid and liquid media used have been described.

2.6. Preparation of culture medium
For isolation of target pathogen *in vitro* condition, potato dextrose agar (PDA) medium was used. For preparation of PDA, 250 g peeled potatoes were cut into slices and boiled in 500ml of distilled water in conical flask. The extract was strained through a piece of muslin cloth and 20 g dextrose was added in it. 20 g agar – agar was melted in 500ml of distilled water separately and was mixed in potato dextrose solution and the volume was made upto 1000 ml by adding distilled water. PDA was poured in flasks, plugged with non– absorbent cotton plugs and sterilized in an autoclave.

2.7. Preparation of slants
For preparation of PDA slants, 2 to 3 ml medium was poured in each culture tube and plugged with non – absorbent cotton and sterilized in an autoclave at 15 lbs pressure p.s.i. for 15 minutes. Later on tubes were kept in slanting position on wooden support and allowed to solidify. Slants were stored in refrigerator. Temporary slides were prepared from pure culture. Calibrated ocular was used for measurement of hyphae, conidia and conidiophores. The length and width of conidia and conidiophores along with width of hyphae were measured with the help of calibrated ocular micrometer.

2.8. Inoculation, incubation and observations
Medium of each flask was poured into 3 petri-plates @ 20 ml per plate, allowed to solidify and inoculated with 5 mm disc of 7 days old culture. Plates were incubated at 28±10 ^{0}C for 7 days and observations were recorded on radial growth and sporulation after 96hrs onwards, respectively.

2.9. Observations on disease intensity were also recorded
2.9.1. Biological studies
Six biocontrol agent *viz*., local isolates of *T. harzianum-1, T. harzianum- 2, T. harzianum-4, T. harzianum-5 Trichoderma viride* and *Trichoderma mutant* were evaluated to test the antagonism against *Fusarium oxysporum* f. sp. *lentis* and *Sclerotium rolfsii.*

2.9.2. Growth of antagonist and the pathogen in dual culture: For screening of the antagonists against *Fusarium oxysporum* f. sp. *lentis* and *Sclerotium rolfsii*, dual culture technique developed by Morton and Straube (1955) [5] was adopted. Twenty ml sterilized melted PDA medium was poured into sterilized Petri plates @ 20 ml/plate aseptically, llowed to solidify, then 6mm discs of the fungus and the antagonistic cut with the help of sterilized cork borer were

placed on PDA approximately 4 cm apart each other and incubated in BOD incubator at 28±1 ºC for 96 hrs. Three replications were maintained for each treatment. Observation on colony diameter of bioagents and test fungus was recorded. Inhibition of mycelial growth of test pathogen over check was calculated by following formula (Vincent, 1947) [12].

Inhibition of pathogen =	Colony diameter of pathogen check in dual culture / Colony diameter of pathogen check in growth (%)	X 100

In order to study the viability of test fungus, reisolation was done by transferring 6mm mycelial disc cut by cork borer from the zone where the test fungus was already overgrown by the antagonist on PDA medium.

2.9.3. Fungicidal studies

In order to find out a suitable fungicides for management of wilt of lentil, six fungicides namely propineb, hexaconazol+zineb, carboxin +thirum along with control were evaluated against *Fusarium oxysporum* f. sp. *lentis* and *Sclerotium rolfsii*, *in vivo* and *in vitro* by following the poisoned food technique. The details about fungicides are given below:

Table 1: Name of fungicides, their doses and formulation

S. No	Name of fungicides	Doses	Formulation
1	Propineb	3.0 g	Powder
2	Hexaconazol+Zineb	3.0 g	Powder
3	Carboxin +Thiram	2.5 g	Powder

2.9.4. Inoculation, incubation and observations

The calculated quantity of fungicides was mixed in the PDA media after sterilization. PDA poisoned with each fungicide was poured into three sterilized Petri-plates @ 20 ml/plate and allowed to solidify. Plates containing PDA without fungicide served as check. After solidification each Petri-plate was inoculated with 5 mm mycelial disc aseptically. Plates were incubated at 28±10 ^{0}C and observation on radial growth of test fungus was recorded after 120 hours and 168 hours. Recorded data on radial growth was converted into per cent growth inhibition by using following formula:

$$\text{Inhibition \%} = \frac{C-T}{C} \times 100$$

Where

C = Colony diameter in check plate (mm)

T = Colony diameter in the treated plate (mm)

Table 2: Categorization of disease intensity (1 – 5 scale)

Scale	Infection type	Lesions amount and location	Disease incidence	Resistant
1.	Very slight to slight infection	One or two few scattered lesions on lower leaves only or clean leaves	Less than 1%	Highly resistant
2.	Light infection	Moderate number of lesions on lower leaves only	1-10%	Resistant
3.	Moderate infection	Abundant lesions on lower leaves and few on middle leaves	11 – 20%	Moderately resistant
4.	Heavy infection	Abundant lesions on lower, middle leaves and extending to upper leaves	21 – 50%	susceptible
5.	Very heavy infection	Abundant lesions on almost all leaves and plants prematurely dry or killed by the disease	More than 50%	Highly susceptible

3. Results and Discussion

The data presented table show that germination per cent of lentil seeds. Significantly increased upon seed dressing with *Trichoderma* strains *viz*. *T. harzianum-1*, *T. harzianum-2*, *T. harzianum-4*, *T. harzianum-5* and *T. mutant* compared to control. Among the *Trichoderma* treatment maximum (74.5%) seed germination per cent was obtained *in T. viride* which was at par with *T. harzianum-5* (72.5%), *T. harzianum-1* (71.5%), *T. harzianum-4* (70.5%) and *T. harzianum-2* (69.45%) as compare to control.

Table 3: Seed germination of lentil in different *Trichoderma* strains and Fungicides.

Treatments	Seed Germination (%)**
T. harzianum-1	71.5 (56.25)**
T. harzianum-2	69.45 (52)**
T. harzianum-4	70.37 (55.5)**
T. harzianum-5	72.5 (57.75)**
T. viride	74.5 (62.75)**
T. mutant	68.5 (54.25)**
Propineb	65.5 (52.25)**
Hexaconazol+Zineb	60.5 (50.5)**
Carboxin +Thiram	62.4 (48.75)**
Control	56.5 (45.25**
SE(m)	2.38
C.D at 5%	6.93

****** Data in parenthesis are angular transformed value.

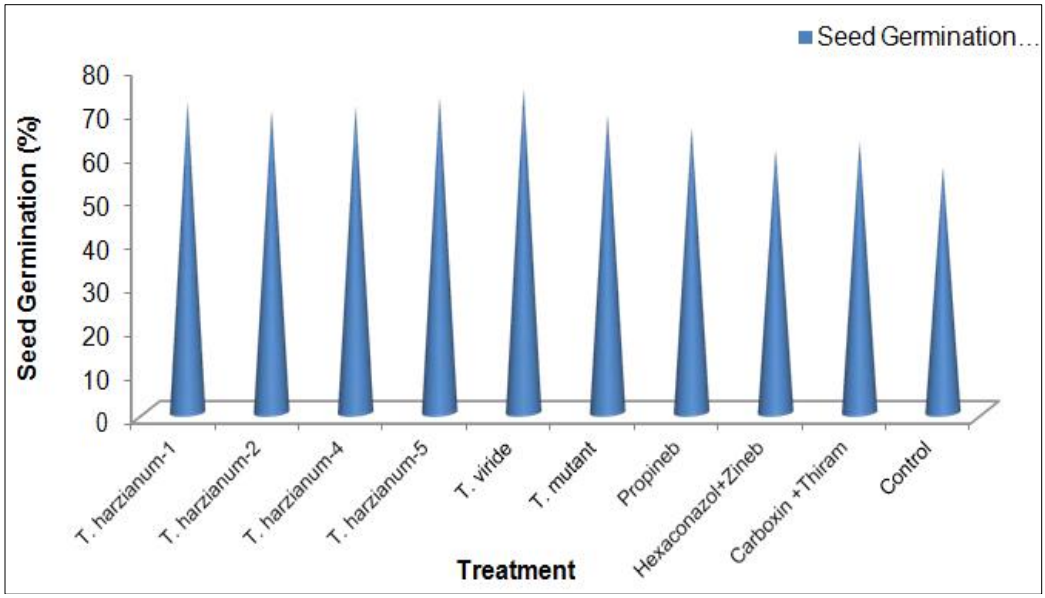

Fig 1: Lentil seed germination in different *Trichoderma* strains and fungicides.

3.1. Incidence of disease

Table 4: Collar rot Incidence in lentil treated with different *Trichoderma* strains and Fungicides.

Treatment	Collar rot Incidence (%)	
	30 DAS	60 DAS
T. harzianum-1	9.5	16.0
T. harzianum-2	11.5	17.5
T. harzianum-4	10.0	18.5
T. harzianum-5	9.25	14.25
T. viride	7.5	13.0
T. mutant	10.5	19.0
Propineb	12.5	20.25
Hexaconazol+Zineb	15.0	20.0
Carboxin +Thiram	12.25	21.5
Control	18.0	32.5
SE(m)	.75	1.29
C.D at 5%	2.19	3.76

Table 5: Wilt Incidence in lentil treated with different *Trichoderma* strains and Fungicides.

Treatment	Wilt Incidence (%)	
	30 DAS	60 DAS
T. harzianum-1	9.87	17.0
T. harzianum-2	11.05	18.20
T. harzianum-4	10.75	17.25
T. harzianum-5	10.0	15.30
T. viride	8.5	14.25
T. mutant	11.25	19.25
Propineb	14.5	21.5
Hexaconazol+Zineb	15.75	23.0
Carboxin +Thiram	16.0	25.25
Control	19.25	35.25
SE(m)	.57	.58
C.D at 5%	1.6	1.7

*Average of 4 replications

3.2. Evaluation of *Trichoderma* strains on growth promoting ability (GPA) of lentil

The data presented in table show the effect of seed dressing with *Trichoderma* strains on shoot and root length, fresh and dry weight, number of nodules, effect on yield components.

Table 6: Shoot and root length in seed dressing of *Trichoderma* strains and fungicides.

Treatment	Shoot length(mm)**		Root length(mm)**	
	30 DAS	60 DAS	30 DAS	60 DAS
T. harzianum-1	11.7	20.6	8.6	11.0
T. harzianum-2	10.8	19.7	8.9	10.5
T. harzianum-4	10.1	22.1	9.2	10.8
T. harzianum-5	12.5	25	9.6	12.1
T. viride	13.4	27.2	10.9	13.9
T. mutant	8.8	20.7	8.7	10.7
Propineb	6.5	19	7.5	10.5
Hexaconazol+Zineb	6.7	18.5	7..0	9.9
Carboxin +Thiram	6.1	17.4	6.7	9.6
Control	5.1	15.3	6.2	8.2
SE(m)	0.36	0.98	0.24	0.21
C.D at 5%	1.5	2.8	0.70	0.62

*Average of 4 replications

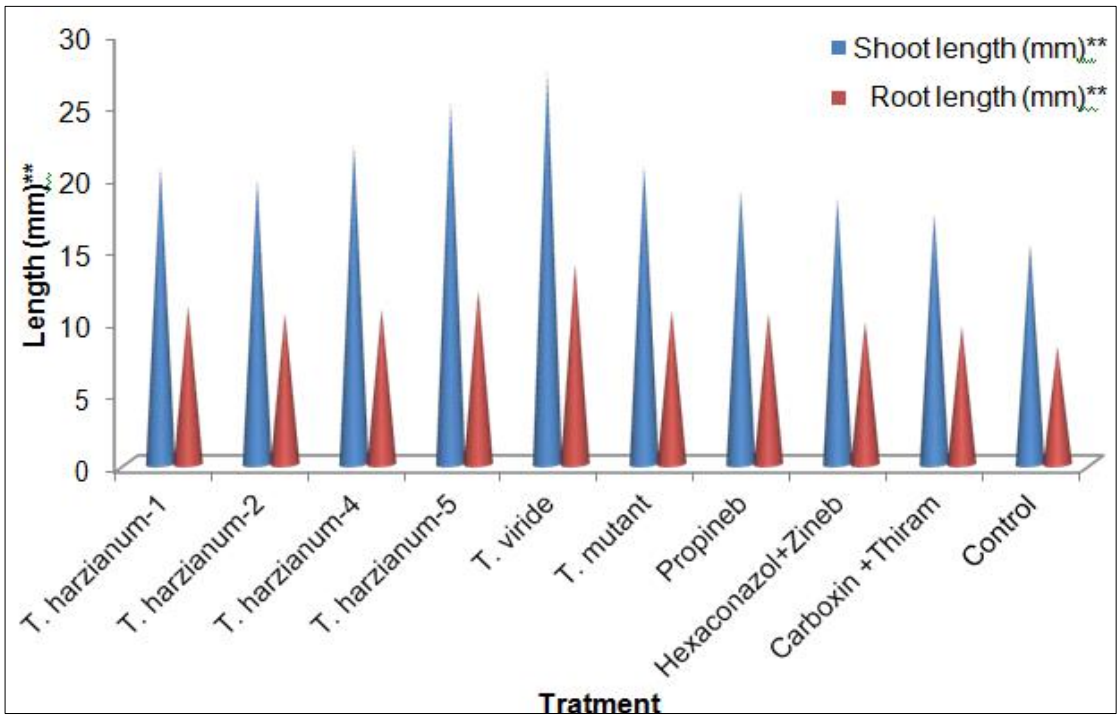

Fig 2: Shoot and root length /plant of lentil in seed dressing with *Trichoderma* strains and fungicides.

3.3. Fresh and Dry weight of Plants

Table 7: Fresh and dry weight in seed dressing of *Trichoderma* strains and fungicides.

Treatment	Fresh weight (g)**		Dry weight(g)**	
	30 DAS	60 DAS	30 DAS	60 DAS
T. harzianum-1	46.0	124.5	23.5	69.2
T. harzianum-2	42.5	125.0	21.5	55.2
T. harzianum-4	41.0	157.5	21.9	56.7
T. harzianum-5	48.0	172.5	25.0	81.7
T. viride	55.7	200	29.2	92.0
T. mutant	40.2	145	21.2	68.0
Propineb	38.7	112	18.0	51.0
Hexaconazol+Zineb	39.2	110	20.7	54.0
Carboxin +Thiram	35.7	95	18.0	46.2
Control	31.0	69	17.2	37.5
SE(m)	1.4	4.38	1.12	3.9
C.D at 5%	4.17	18	3.19	11

*Average of 4 replications.

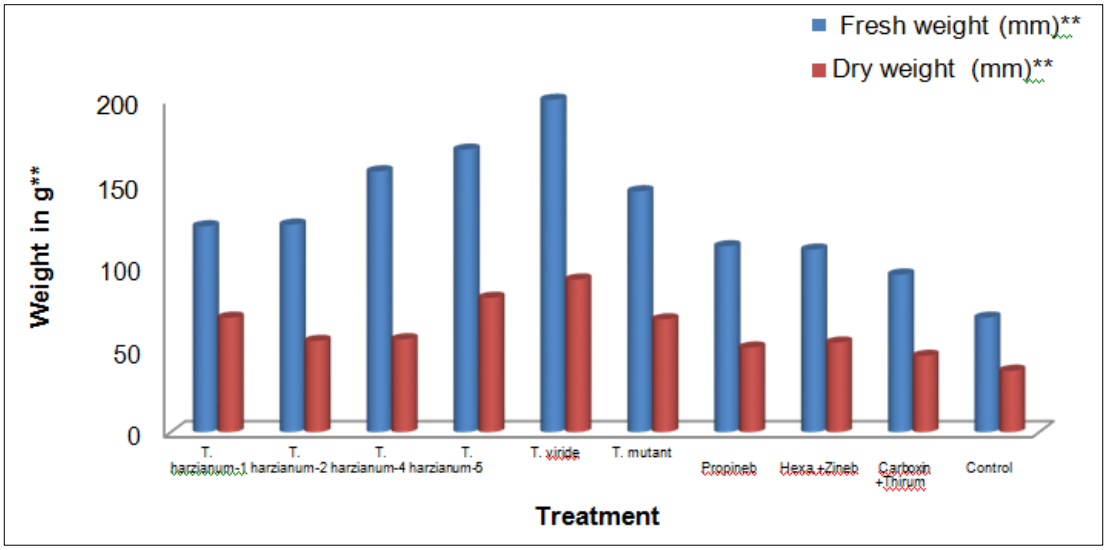

Fig 3: Fresh and dry weight/plant of lentil in seed dressing with *Trichoderma* strains and fungicides.

3.4. Number of nodules

Table 8: Number of nodules/plant in lentil in seed dressing with *Trichoderma* strains and fungicides.

Treatment	Number of nodules/plant	
	30 DAS	60 DAS
T. harzianum-1	8.9	10.8
T. harzianum-2	8.7	11
T. harzianum-4	8.5	10.9
T. harzianum-5	9.9	12.6
T. viride	10.7	14.4
T. mutant	8.4	10.5
Propineb	7.0	10.1
Hexaconazol+Zineb	7.8	9.2
Carboxin +Thiram	6.9	10.2
Control	5.9	8.6
SE(m)	0.4	0.34
C.D at 5%	0.82	1.0

*Average of 4 replications

Among the *Trichoderma* treatment, *T. viride* (10.7) higher number of nodules /plant was obtained followed by *T. harzianum-5* (9.9) and *T. harzianum-1* (8.9) as compared to control. Significantly higher number of nodules/plant were also observed after 60 DAS of sowing in all the *Trichoderma* strains compared to control. While number of nodules /plant of *T. viride* (14.4) obtained higher nodules/plant was observed. Seed treatment with *Trichoderma* strains increased in shoot length/plant as compared to control. Significantly higher shoot length was observed in the *T. viride* (27.2g) followed by *T. harzianum-5* (25.0g) as compared to control. Similarly, significantly higher fresh weight was observed in the *T. viride* (200g) and *T. harzianum-5* (157g) as compared to control. Increase in dry weight of *Trichoderma* treated plants establishes the positive effect of the bio-control agents on enhancement of plant growth. Vinale *et al*. (2008) [11] suggested that certain secondary metabolites produced by *Trichoderma* exert an antimicrobial effect at high doses and also seed treatment of *Trichoderma* strains act as MAMPs and as auxin-like compounds at low concentrations. At 1 p.p.m., 6-pentyl-a-pyrone, harzianolide and harzianopyridone activate plant defence mechanisms and regulate plant growth in pea, tomato and canola. Singh (2010) [8] reported that *Trichoderma* enhances yield along with quality of produce, boost germination rate. Increase in shoot & root length solubilizing various insoluble forms of phosphates augment nitrogen fixing and promote healthy growth in early stages of crop. Increase dry matter production substantially. Provide natural long term immunity to crops and soil. Number of nodules were enhanced by biological control agents *Bacillus thuringienesis* and *T. harzianum* (Mark, 2012) [4]. Significantly increase in number of nodules /plant was observed in seed dressing with *Trichoderma* strains *viz. T. viride*, *T. harzianum-1*, *T. harzianum-2*, *T. harzianum-4*, *T. harzianum-5* and *Trichoderma mutant* compared to control. Among the *Trichoderma* treatment, *T. viride* had higher number (14.4) of nodules /plant followed by *T. harzianum-5* (12.6), *T. harzianum-4* (10.9)as compared to control followed by propineb, hexaconazol+zineb, carboxin + thiram.

Benitez *et al.*, (2004) [1] found that *Trichoderma* strains establish long-lasting colonization of plant roots and penetrate into the epidermis. there, they produce or release compounds that induce localized or systemic plant resistance responses. Plants react against fungal invasion by synthesizing and accumulating phytoalexins, flavonoids and terpenoids, phenolic derivatives, aglycones and other antimicrobial compound. *Trichoderma* strains can interact directly with roots, increasing plant growth potential, resistance to disease and tolerance to abiotic stresses. Seed treatment with *Trichoderma* strains *viz.*, *T. harzianum-1*, *T.*

harzianum-2, *T. harzianum-4*, *T. harzianum-5*, *T. viride* and *Trichoderma mutant* increased the yield and yield components. Significant maximum number of pods was recorded in *T. viride* (58.1), followed by *T. harzianum-5* (47.2), and *T. harzianum-2*(44.2), as compared to control.. Significantly increase in yield(kg/ha) was noticed in plants treated with *Trichoderma* strains *T. viride* (468.8kg/ha), followed by *T. harzianum-5* (387.9 kg/ha), *T. harzianum-4* (385.1kg/ha) as compared to control. The Disease Incidence of collar rot and wilt in lentil was significantly less in *Trichoderma* strains treated seeds as compared to control. seed treatment with *T. viride* (13% &14.25%) was the best in controlling collar rot and wilt of lentil. followed by *T. harzianum-5* (14.25% &15.3%) as compared to other treatment. These result are in agreement with the findings of Solanki *et al*., (2011) [10] who reported that the fruit yield of treated plant with *Trichoderma* were 66 g per plant, where as the yield was only 41 g for a control Kashem *et al.*, (2011) [2] also showed that seed treatment plus soil application of talc based formulation of *T. viride* isolation *Tv*1 and *Tv*2 along with FYM effectively reduced the disease incidence and increased the yield of black gram.

4. Conclusion

From evaluation of *Trichoderma* strains for bio-efficacy, compatibility with fungicides and growth promoting ability (GPA) of lentil, it could be concluded that *Trichoderma* strains *viz*. *T. virie and T. harzianum-5*. Among the *Trichoderma* strains *T harzianum-5* and *T. harzianum-4* were highly compatible with hexaconazol+zineb, while strains *T. harzianum-2*, *T. harzianum-4* were compatible with carboxin + thiram. *Trichoderma* strains *T. viride* increased growth of lentil plant followed by other *Trichoderma* strains *viz. T.harzianum-5, T.harzianum-4, T. harzianum- 2* and *T. mutant.*

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