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Ghanshyam Kumar Pandey
Research Scholar, Department of Plant Pathology, Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj, Uttar Pradesh, India

Shafaat Ahmad
(Retd. Professor and Ex Head, Department of Plant Pathology) Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj, Uttar Pradesh, India

Bhushan Kewate
Department of Plant Pathology, RajmataVijayaraje Scindia Krishi Vishwavidyalaya, Gwalior, Madhya Pradesh, India

Anoop Yadav
Department of Plant Pathology, Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj, Uttar Pradesh, India

Brajendra Kumar Yadav
Additional Commissioner, Government of India, Ministry of Agriculture & Farmers' Welfare, Krishi Bhawan, New Delhi, India

Sunil Zacharia
Department of Plant Pathology, Faculty of Agriculture, Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj, Uttar Pradesh, India

Gaurav Chaudhary
Department of Horticulture, Chaudhary Charan Singh University, Meerut, Uttar Pradesh, India

Nikunj Tyagi
Department of Agriculture Extension, Chaudhary Charan Singh University, Meerut, Uttar Pradesh, India

Pradeep Kumar Shukla
Department of Biological Sciences, Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj, Uttar Pradesh, India

Corresponding Author:
Ghanshyam Kumar Pandey
Research Scholar, Department of Plant Pathology, Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj, Uttar Pradesh, India

Shelf life studies of some bio-pesticide formulations and their efficacy against chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceri*

Ghanshyam Kumar Pandey, Shafaat Ahmad, Bhushan Kewate, Anoop Yadav, Brajendra Kumar Yadav, Sunil Zacharia, Gaurav Chaudhary, Nikunj Tyagi and Pradeep Kumar Shukla

Abstract
Evaluate the shelf life and prepare the formulation of the isolated *Trichoderma viride*, *Trichoderma harizianum*, and *Beauveria bassiana*. *In vivo* conditions soil inoculated with *Fusarium oxysporum* f. sp. *ciceri* was conducted and compare the efficacy of different treatments *viz.* Seed treatment with commercial formulation of bio-control agents and fungitoxicants in the management of Gram (chickpea) wilt. The two year pooled data of *Trichoderma viride Trichoderma harzianum Beauveria bassiana* At 10^6 (D_1) and 10^7 (D_2) dilution factor was found to be significant and data indicate consequently decline in the order of cfu/g during the year of 2015-16 and 2016-17, average of both year. In *Beauveria bassiana* some viable propagules seen during the experiment. So it might be decline due to lesser no of viable propagules in talc based formulation of *Beauveria bassiana.* Inhibitory effect of *Trichoderma* sp. against radial growth of growth of test fungus reported highest *(*42.67 mm) in control T_0 highest percentage of inhibition was found in T_3 *Trichoderma harizianum, Trichoderma viride* (T_1). *Trichoderma viride* was found to be significantly superior over *Trichoderma harzianum.* The data suggested that selected bio-pesticide have potential to control Chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceri* and therefore could be effectively utilized in eco-friendly management of Chickpea wilt**.**

Keywords: *Pseudomonas Fluorescens*, *Trichoderma viride* Fungitoxicants, *Fusarium oxysporum* f. Sp. *ciceri*

Introduction
Chickpea (*Cicer arietinum* L.) is an important pulse crop of family *Leguminaceae*. It is used as a big source of protein in the human diet. Chickpea is one of the best legumes for human consumption. Chickpea was originated from West Asia and is now cultivated in 55 countries of the world. Worldwide it is grown on an area of 13.5 million hectares with a production of more than 13 million tons. It is an important crop of Indian sub-continent that usually contributes more than 66% in terms of global production, while In India, chickpea is ranked first in terms of production and consumption in the world. About 65% of global area with 68% of global production of chickpea is contributed by India (Amarender and Devraj, 2010) [1]. Low yield of chickpea is attributed to its susceptibility to several fungal, bacterial and viral diseases. *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *ciceri*, is the most important soil-borne disease of chickpea throughout the world and particularly in the Indian Subcontinent (Nene and Reddy, 1987) [37].

At the national level, chickpea yield losses encounter due to wilt may vary between five to ten percent (Dubey *et al*., 2007) [12]. *F. oxysporum* f. sp. *ciceri* infects chickpea at seedling as well as at flowering and pod forming stage (Grewal, 1969) [18], with more incidence at flowering and podding stage. *F. oxysporum* f. sp. *ciceri* is a facultative saprophytic and it can survive as mycelium and chlamydospores in seed, soil and also on infected crops residues, buried in the soil for up to five to six years (Haware *et al*., 1986) [22]. Therefore, integrated disease management strategies are the only solution to maintain plant health. These strategies should include minimum use of chemicals for checking the pathogen pollution, encouragement of beneficial biological agents to reduce pathogen inoculum, modification of cultural practices and use of resistant varieties (Bendre and Barhate, 1998) [2]. *Trichoderma* spp. generally grows in its natural habit on plant root surface and therefore it controls root diseases in particular (Monte, 2001; Faruk *et al*., 2002; Kamlesh and Gujar, 2002) [16, 15, 29].

The species of *Trichoderma* have been evaluated against the wilt pathogen and have exhibited greater potential in managing chickpea wilt under field condition (Podder *et al*., 2004) [39]. The disease can appear at any stage of plant growth, symptoms in a highly susceptible cultivar can develop any time between 25 days after sowing till as late as podding stage (Nene 1985) [38]. Annual yield losses in chickpea were estimated to be 4.8 million tones worldwide due to biotic stresses, including infectious plant diseases (Ryan, 1997) [42].
In India it is 10–15%, which in years of severe epidemics may rise to 60–70% (Jalali and Chand, 1992) [28]. Mycoparasites and many bacteria have shown promising results in managing phytopathogenic fungi. *P. fluorescens* has revolutionized the field of biological control of soil-borne plant pathogenic fungi (Burr *et al*., 1998) [4]. That bacterium produces phenazin (Toohey *et al*., 1965; Gurusiddaiah *et al*., 1986) [4, 19], pyrolnintrin (Burkhead and Geoghegan, 1994) [5], phloroglucinol (Howell and Stipanovic, 1980) [25] and siderophores (Sakthivel *et al*., 1986) [44], which may be involved in the suppression of the wilt fungus (Fridlender *et al*., 1993; Gamliel and Katan, 1993) [17, 20]. Leeman *et al*. (1995) [31] reported satisfactory control of Fusarium wilt of radish by treating the seed with *P. fluorescens.* In addition, *P. fluorescens* produces auxins, gibberellins etc. (Glick, 1995) [21] and solubilises phosphorus in the soil (Dube and Yeole, 1997) [13], which helps plant growth. Among my-coparasites, the genus *Trichoderma* includes the most widely used biocontrol agent of soil-borne, seed-borne and other diseases (Chet *et al*., 1979; Chet and Baker, 1981) [9]. *Trichoderma harzianum* and *T. virens* are active rhizosphere colonisers (Tronsmo and Harman, 1992) [47] that produce antibiotics such asgliotoxin, viridin, and some cell wall degrading enzymes (Larito *et al*., 1976; Bello *et al*., 1997) [32, 6] and also certain biologically active heat-stable metabolites such as ethyl acetate (Claydown *et al*., 1987) [11]. These substances may be involved in disease suppression or plant growth promotion. *Trichoderma harzianum* is one efficient biocontrol agent that is successfully used to suppress Fusarium wilt (Khan *et al*., 2004; Dubey *et al*., 2007) [30, 14]. Similarly, amending soil with plant extracts significantly reduces Fusarium wilt in the field (Chand and Singh, 2005) [10]. In view of above a laboratory study carried out to examine the shelf life and prepare the formulation of the isolated *Trichoderma viride*. To study the effect of fungicides on disease incidence. To study the effect of fungicides on test fungus. To study the effect of bio control agents on disease incidence

Materials and methods

There are three types of biocontrol agents used for control of Chickpea wilt *viz. Trichoderma harzianum Trichoderma viride & Beauveria bassiana* on test fungus *Fusarium oxysporum* f. sp. *ciceri*. Isolation of *Trichoderma* spp. was done from soil by serial dilution and plate count method described by Johnson *et al*. 1959. 10g Rhizosphere soil was added in 100ml sterilized water blank and was shaken well for 15minutes. Serial dilutions were prepared to be 10^{6} by adding 1ml of 10^{-6} dilution was transferred, melted and cooled TSM was poured in each petriplate. The plates were rotated gently and allowed to solidify and incubated at room temperature for 5-6 days when *Trichoderma* colonies were observed. The identification of *Trichoderma* spp. was done on the basis of colony characteristics and microscopic examination. Standard book and papers were consulted while the examination of these fungi (Aneza, 2004; Rifai, 1969; Barnet and Hunter, 1999). Persistence conidiophores hyaline much branched, not verticillate phiallides single or in small terminal clusters usually easily recognized by its rapid growth and green patches or cushions of conidia saprophytic on soil or on wood (Barnet and Hunter, 1999). The culture of *Trichoderma* spp. was purified from the isolated petriplates and maintained by periodic sub-culturing inb TSM petriplates, TSM slants and TSM broth. Sterilized cork borer was used to cut 5 mm. diameter discs from actively growing fungus culture and transferred aseptically in thecentre of petridishes and slants containing solidified TSM. With the help of sterilized cork borer cut the 5 mm diameter discs of the *Trichoderma* fungi were inoculated in the conical flasks containing TSM broth. After transferring the fungus all the conical flasks were kept at room temperature and the culture was observed after 5 days (Saode *et al.*, 1998). Respective species of *Trichoderma* were ground in mixer, I liter content was uniformly mixed with 2 kg of talcum powder (1:2 ratio), maintaining 8-10% moisture, CMC (Carboxy methyl cellulose) was added @ 0.5% and packet in bags. These were used for shelf-life studies of formulation.

Table 1: Characters of *Trichoderma harizianum* and *Trichoderma* viride (Rifai; 1969)

S. No	Characters	***Trichoderma harizianum***	***Trichoderma viride***
1	Perfect Stage	*Hypocre alboufulba*	*Hypocre rufa*
2	Branching system	Regular	Irregular
3	Odour culture	----------	Coconut odour at matiurity
4	Phialides	Short crowded and regularly 5-7 X 3-3.5	Long and irregular 8-14X2.4-3
5	Spore Shape	Sub globose with smooth wall	Globose with irregular wall
6	Spore size	Smaller 2.8-3.2X2.5-2.8	Larger 3.6-4.8X3.5-4
7	Spore orientation	Absent	Present

Collection

The best season for collection of fungal infected insects from different crops/regions was August-September during which fungal infection on various crop pests were common due to the prevalence of high humidity and favorable temperature. Mycelium and spores from fresh infected insect guava caterpillar larvae specimen were placed directly on SDA in sterilized petriplates which were incubated at 26$\pm$2^{0}Ctemperature. *Beauveria* colonies were observed after 5-6 days (Jhonsan *et al.*, 1959). Colony white, hyphae cylindrical 3.5 wide hyaline, septate, conidiophores single or branched, abundant arising from vegetative cells globose to flask shaped (3-5 X 3-7 m) with well-developed rachis up to 20 m long and 1-1.5 m wide, conidia were borne at thread like apex of the phialide on a series of zig-zag branch lets, more or less comparable to a cyme, conidia globose (1-4 to oval (1.5-5 X1.0-3.0), smooth and hyaline (Aneja, 2004 and Barnet and Hunter, 1999).

Maintenance and Multiplication of pure culture

The cultures of *Beauveria bassiana* was maintained by periodic sub-culture in slants/petriplate in SDA/PDA slants. With the help of sterilized cork borer cut the 5 mm diameter discs of the *Beauveria bassiana* was inoculated in the conical flasks containing SD/PD broth. After transferring the fungus all the conical flasks were kept at room temperature (26 ± 2^0C) and the culture was observed after 5 days).Respective isolates were grounded in mixer and 1 litre content was mixed uniformly in 2 kg of talcum powder (1:2 ratios) maintenance 8-10% moisture and CMC @ 0.5% concentration. Requited 90ml of water were taken into clean conical flasks and were autoclaved at 1470 g/cm for 20 minutes during serial dilution and plate count method (Johnson *et al*., 1959), cfu of respective formulations of *Trichoderma harzianum, Trichoderma viride and Beauveria bassianba* were estimates at monthly interval from 0 day to 7 months (Aneja, 2004). The flask containing the 10 g of biopesticide formulation added into 90 ml sterilized water and was shake well and serially diluted $1X10^{-6}$ to $1X10^{-7}$ one ml of respective dilution was placed on identical media. After 5 days total number of colonies was recorded in case of cfu calculated as below:

Cfu/g=No. of colonies x dilution factor

Test pathogens *Fusarium oxysporum* f. sp. *ciceri* multiplied on sorghum medium. 100 gm of sorghum was crushed and soaked overnight, moisture was adjusted to 10% 100 gm of sorghum was taken in conical flasks and were sterilized in an autoclave at room temperature 121ºC temperature and 15 lbs pressure for 20 minute. Inoculated each conical flask with carried sorghum with 2 disc measuring 5mm of *Fusarium oxysporum* f. sp. *ciceri* and incubated at 25ºC (Jhonson *et al*., 1999). The required quantity of *B. bassiana* formulation was measured and first mixed with small quantity of water and later made up to get the required volume of spray fluid. The spray fluid was stirred thoroughly before spraying. Adjuvant like 1% jiggery and pinch of robin blue were added to *B. bassiana* formulation. The spraying was given during evening when the weather was still. A foot sprayer was used to applying the *B. bassiana* formulation. The plants were covered with the spray fluid thoroughly to the point of run-off. Each plot (4.5 M^2) received 0.5 Lt. of spray fluid. The different treatments and dilutions made for preparing spray fluids are given the table. Seed were dressed (as per the treatment) with formulation of *Trichoderma viride* and *Trichichoderma harzianum* 4 g/kg of seeds The fungus was removed from the flask and then pressed in between the blotting paper then it was weighed for preparing different concentration (weight/volume) of 2%, 4%,6% and 8% respectively i.e.,2% lab formulation: 2 gm *Beauveria bassiana* net in 98 ml distilled water; 4% lab formulation 4 gm *Beauveria bassiana* net in 96 ml distilled water; 6% lab formulation: 6 gm *Beauveria bassiana* net in 94 ml distilled water; 8% lab formulation, 8gm *Beauveria bassiana* net in 92ml distilled water The pathogen *Fusarium oxysporum* f. sp. *ciceri* was culture on sorghum medium and was applied @ 10g/plot(Aneja, 2004 and Barnett and Hunter 1999).

Result and Discussion

The shelf life of biopesticide is judged by number of viable spores present in formulation on a particular point of time. Thus quality of biopesticide in market is determined by number of cfu (Colony Forming Unit).

Under laboratory conditions (*In – vitro*)

Trichoderma viride

At 10^6 dilution factor (D_1)

It was observed from two years (2015-16 and 2016-17) pooled data that from September 2015 to April 2016 shelf life period in talc based formulation of *Trichoderma viride* at 10^6 dilution factor (D_1) was found to be significant. The Mo (Zero days) show maximum number of cfu/g *Trichoderma viride* colonies (70.67) in talc based formulation at10^6 dilution factor followed by M_1 (62.33), M_2 (49.33), M_3 (29.33), M_4 (16.00), M_5 (8.33), M_6 (6.33) and M_7 (0.67). Further it was found that the inter-relationship with other months *viz*. M_o, M1, M2, M3 and M_4 was found to be significant in talc based formulation of *Trichoderma viride* at 10^6 dilution factor ($D_{1)}$. It was observed that during (2015-16 and 2016-17) there was decline in cfu/g i.e.$T_0>T_1>T_2>T_3>T_4>T_5>T_6>T_7$.

At 10^7 dilution factor (D_2)

The perusal of two years (2015-16 and 2016-17) pooled data shows that from September 2015 to April 2016 shelf life period in talc based formulation of *Trichoderma viride* at 10^7 dilution factor (D_1) was found to be significant. Maximum number of *cfu/g Trichoderma viride* in talc based formulation at10^7 dilution factor D_2 was observed in M_0 (7.00) followed by M_1(6.00), M_2 (4.73), M_3 (2.90), M_4 (1.83), M_5 (0.77), M_6(0.50) and M_7 (0.07). The monthly interval relativity between M_5 with M_6 and M_6 with M_7 showed non-significant results, whereas their inter-relationship with other months *viz*. M_o, M_1, M_2, M_3 and M_4 was found to be significant at 10^7 dilution factor (D_2).The result further indicate decline in the order of cfu/g during (2015-16 and 2016-17) i.e.$T_0>T_1>T_2>T_3>T_4>T_5>T_6>T_7$. Our result are agreement with Ramkrishnan *et al.* (1994) and Jayarajan and Nakeeran (1996). Talc based formulation of *Trichoderma* spp. Retained significantly high viable propagules at 180 days. Jeyarajan and Nakeeran (1996) reported that *Trichoderma viride* talc based formulation viability from 230-242X10^6 at ambient temperature till 120 days.

Table 1: cfu/g in talc based formulations for *Trichoderma viride*

Month	*Trichoderma viride*		*Trichoderma harzianum*		*Beauveria bassiana*	
	10^6	10^7	10^6	10^7	10^6	10^7
September	70.67	7.00	78.33	7.83	36.33	3.67
October	62.33	6.00	63.33	6.33	26.33	2.83
November	49.33	4.73	49.00	4.90	20.00	1.83
December	29.33	2.90	29.67	2.83	16.67	1.60
January	16.00	1.83	13.00	1.33	11.33	1.07
February	8.00	0.77	5.33	0.50	7.33	0.70
March	6.33	0.50	3.33	0.30	4.00	0.30
April	0.67	0.07	0.67	0.07	0.67	0.07
	S. Ed. (+) 2.120 C.D. at 5% 4.548	S. Ed. (+) 2.42 C.D. at 5% 0.518	S. Ed. (+) 2.157 C.D. at 5% 4.626	S. Ed. (+) 20.237 C.D. at 5% 0.509	S. Ed. (+) 1.233 C.D. at 5% 2.644	S. Ed. (+) 0.0263 C.D. at 5% 0.564

Table 2: Inhibitory effect of *Trichoderma* spp. against Radial growth (in mm) of *Fusarium oxysporum* f. sp. *ciceri* at different interval

Treatments	24 hrs		48 hrs		72 hrs		Over all % ioc
	Mean	% IOC	Mean	% IOC	Mean	% IOC	
T_1	10	11.76	11.67	25.53	12	43.75	27.01
T_2	9.67	14.71	12.33	21.27	14.33	32.81	22.93
T_3	7.67	32.35	9.00	42.55	10.00	53.12	42.67
T_0	11.33	0.00	15.67	0.00	21.33	00.00	
	S. Ed. (+) 0.707 C.D. at 5% 1.541		S. Ed. (+) 1.155 C.D. at 5% 2.516		S. Ed. (+) 1.269 C.D. at 5% 2.766		

Trichoderma harzianum

At 10^6 dilutions factor (D_1)

The data indicate that from September 2015 to April 2016 shelf life period in talc based formulation of *Trichoderma harzianum*at 10^6 dilution factor (D_1) was found to be significant. Maximum number of cfu/g *Trichoderma harzianum* in talk based formulation at10^6 dilution factor (D_2) was observed in M_0 (78.33) followed by M_1 (63.33), M_2 (49.00), M_3 (29.70), M_4 (13.00), M_5 (5.33), M_6 (3.33) and M_7 (0.67). Further it was found that the inter-relationship between $M_5$with M_6 and M_6 with M_7 showed non-significant result in talk based formulation at 10^6 dilution factor ($D_{1)}$. It was observed that during (2015-16 and 2016-17) there was decline in cfu/g i.e.T_0>T_1>T_2>T_3>T_4>T_5>T_6>T_7.

At 10^7 dilution factor (D_2)

The perusal of two years (2015-16 and 2016-17) pooled data showed that during September 2015 to April 2016 shelf life period in talc based formulation of *Trichoderma harzianum* at 10^7 dilution factor (D_2) was found to be significant. A dilution factor (D_2) Maximum number of cfu/g *Trichoderma harzianum* in talc based formulation was observed in M_0 (7.83) followed by M_1 (6.33), M_2 (4.90), M_3 (2.83), M_4 (1.33), M_5 (0.5), M_6 (0.30) and M_7 (0.07). The inter relationship between M_5 with M_6 and M_6 with M_7 showed non-significant results, whereas their inter-relationship with other months *viz*. M_o, M1, M2, M3 and M_4 was found to be significant in talc based formulation of *Trichoderma harzianum* at 10^7 dilution factor (D_2).The result further indicate decline in the order of cfu/g during (2015-16 and 2016-17) i.e.T0>T_1>T_2>T_3>T_4>T_5>T_6>T_7.

At 10 7 dilution factor (D_2) number of *Trichoderma harzianum* colonies was recorded more during 0-120 days (M0 to M4). Our results are with the conformity of Prasad and Rangeshwaran (2000) they reported that cfu/g in talc based formulation of *Trichoderma harzianum* was estimated to be more at 10^6 and 10^7 dilution factors during 0-120 days and there was significant decline in *Trichoderma harzianum* colonies during monthly interval from 0 days to 210 days.

Beauveria bassiana

At 10^6 dilutions factor (D_1)

Appraisal of the pooled indicate that from September 2015 to April 2016 shelf life period in talc based formulation of *Beauveria bassiana* at 10^6 dilution factor (D_1) was found to be significant. M_0 (zero days) shows Maximum number of cfu/g *Beauveria bassiana* (36.33) in talc based formulation at10^6 dilution factor followed by M_1 (26.33), M_2 (20.00), M_3 (16.67), M_4 (11.33), M_5 (7.33), M_6 (4.00) and M_7 (0.67). Further it was found that the inter-relationship between M_5 with M_6 and M_6 withM_7 showed non-significant result in talc based formulation of *Beauveria bassiana* at 10^6 dilution factor ($D_{1)}$. However their inter relationship with other months *viz*. M_o, M1, M2, M3 and M_4 was found to be significant in talk based formulation of *Beauveria bassiana* at 10^6 dilution factor (D_1). It was observed that during (2015-16 and 2016-17) there was decline in cfu/g i.e.T_0>T_1>T_2>T_3>T_4>T_5>T_6>T_7.

At 10^7 dilution factor (D_2)

The perusal of two years (2015-16 and 2016-17) pooled data showed that during September 2015 to April 2016 shelf life period in talc based formulation of *Beauveria bassiana* at 10^7 dilution factor (D_2) was found to be significant. A Maximum number of cfu/g in talc based of *Beauveria bassiana* at 10^7 dilution factor (D_2) was observed in M_0 (3.67) followed by M_1 (2.83), M_2 (1.83), M_3 (1.60), M_4 (1.07), M_5 (0.70), M_6 (0.30) and M_7 (0.07). The inter relationship between M_5 with M_6 and M_6 with M_7 showed non-significant results, whereas their inter-relationship with other months *viz*. M_o, M1, M2, M3 and M_4 was found to be significant in talc based formulation of *Beauveria bassiana* at 10^7 dilution factor (D_2).The result further indicate decline in the order of cfu/g during (2015-16 and 2016-17) i.e.T_0>T_1>T_2>T_3>T_4>T_5>T_6>T_7.

This significant decline might be due to presence of lesser no of viable propagules in in talc based formulation of *Beauveria bassiana*. Puzari *et al.* (1997) reported that mass culture of *Beauveria bassiana* in a solid medium composed of rice hull, saw dust and rice brawn and could harvest 33X10^7conidia /ml. Our result is closely agreement with Sandhu *et al.* (1993), Moore & Higgins (1997 and Nirmala *et al*. (2005).

Inhibitory effect of *Trichoderma* spp. Against Radial growth (in mm) of *Fusarium oxysporum* f. sp. *ciceri*

It is revealed that during both the year of investigation *Trichoderma harzianum* (T_1), *Trichoderma viride* (T_2) and their combination (T_3) was resulted reduction in the growth of *Fusarium oxysporum* f. sp. *ciceri*. The growth of test fungus was reported highest (42.67 mm) in control (T_0). The highest percentage of inhibition was found in (T_3) (*Trichoderma harzianum* (T_1), *Trichoderma viride)* that was applied at different intervals i.e. 32.35% at 24 hrs, at 42.55% at 48 hrs and 53.12% at 72 hrs respectively. This was followed by T_1 and T_2. *In vitro* studies of *Trichoderma* s.p in solid medium during both the years of investigation significantly inhibited the growth of *Fusarium oxysporum* f. sp. *ciceri*. similar findings that *in vitro* condition *Trichoderma viride* highly inhibit and suppress the growth of *Fusarium oxysporum* f. sp. *ciceri* and further *Trichoderma viride* was found to be significantly superior over *Trichoderma harzianum.*

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