

Neem cake carrier prolongs shelf life of biocontrol fungus *Trichoderma viridae*

VP Zope¹, HP Jadhav & RZ Sayyed^{2*}

¹Department of Microbiology, DNCVPs, SM Chaudhari College, Jalgaon, Maharashtra-425 002, India

²Department of Microbiology, PSGVP Mandal's Arts, Science, and Commerce College, Shahada, Maharashtra-425 409, India

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Sustainable increase in agricultural productivity to feed the growing population demands eco-friendly remedies including the use of biocontrol agents to control diseases in food crops. *Trichoderma* spp. is one such agent which provides multiple plant health benefits, like disease control, plant growth promotion, development of resistance and stress tolerance. However, inappropriate concentration and less effective formulations with a poor shelf life of *Trichoderma* spp. have hampered its customized applications at large scale. Here, we studied the influence of various parameters on growth and mass production of customized applications of *Trichoderma* spp., including carrier materials, its shelf life and evaluation of antifungal activity of *T. viridae*. We observed optimum growth of *T. viridae* at 40°C, pH 6.0, maximum sporulation at 45°C and pH 6.0, with cellulose and carboxymethyl cellulose as a best utilizable carbon source. Neem cake came out as an excellent carrier as it gave a prolonged shelf life of 200 days during storage at 28°C with 35.78×10^6 cfu g⁻¹ of *T. viridae*. Antifungal assay against plant pathogenic fungi revealed complete inhibition of growth and sporulation of fungal pathogens.

Keywords: Antifungal, Biocontrol agent

Trichoderma viridae is an effective biocontrol agent as well as plant growth promoting fungus (PGPF). It prevents the growth of phytopathogens by the mechanism of rhizosphere competence, mycoparasitism¹⁻⁴ through hydrolytic enzymes like cellulase⁵, chitinase⁶⁻⁸, glucanase⁹ and induced systemic resistance mechanism in plants¹⁰⁻¹³. It provides phytohormones, phosphate, mineral and germination stimulants to crop plants¹³.

Use of *Trichoderma* spp. as BCA offers many advantages, the fungus can be easily grown on a wide variety of substrates thus can be easily cultivated for large scale production. It produces metabolites with proven antibiotic and mycoparasitic activity against a

wide variety of plant pathogens, it induces changes in rhizospheric microflora with stabilization of soil nutrients, thereby promoting sufficient growth of roots and root hairs¹². For fruitful application of *T. viridae* as biocontrol agent and PGPF under natural soil conditions, it is necessary to study the influence of abiotic stress factors like temperature and pH which affect the growth and sporulation of fungus¹⁴.

Traditionally used synthetic media like glucose, cellulose, soluble starch, etc. and carrier materials like the use of pyrax, talc and alginate does not offer cost effective formulation and suffers from poor shelf life of formulation¹⁵. Hence, use of cost effective substrates, such as cellulosic waste material viz. rice bran, corn bran and wheat bran for cultivation of *Trichoderma* spp. and sustainable carriers like neem cake, charcoal powder, as a carrier material for formulation are economical, eco-friendly, and also offers good shelf life during storage and also maintain the high propagule count during storage. In the present study, we explored the use of cellulosic waste as a substrate for cultivation of *Trichoderma* spp., optimized growth conditions, and also evaluated neem cake, charcoal, etc. as carrier material as well as the antifungal activity of fungus against common phytopathogenic fungi.

Materials and Methods

Trichoderma viridae was procured from National Chemical Laboratory (NCL), Pune, India. It was immediately sub-cultured on sterile Potato Dextrose Agar (PDA) slants and stored under refrigeration conditions until further use. *Rhizoctonia solani* and *Fusarium oxysporum* were procured from Krishi Vigyan Kendra (ICAR-KVC) Jalgaon, Maharashtra, India.

T. viridae was grown in 100 mL Potato Dextrose Broth (PDB) for 6 days at 28°C, the concentration of conidia was adjusted with the help of hemocytometer slide and few drops of tween 80 were added to the broth to ensure homogeneity in conidia distribution. A 100 mL of this conidia preparation (10^8 conidia mL⁻¹) was transferred to one liter of sterile PDB and incubated at 28°C for 8 days under shaking condition (100 rpm).

Uniformly grown culture plates were selected as inoculum for studying the effect of different

*Correspondence:
E-mail: sayyedrz@gmail.com

temperatures on the growth of *T. viridae*. Approximately, 50 mm growth of *T. viridae* was aseptically removed from each plate with the help of cork borer and aseptically separately placed in the center of PDA plate and incubated at 15, 20, 25, 30, 35, 40 and 45°C and observed for growth. Growth was measured after every 24 h up to 6 and expressed in terms of radial growth and dry weight¹⁵. PDA medium separately prepared with a pH range of 4-8 was individually grown with 50 mm well-grown agar disc of *T. viridae* for 6 days at 28°C¹⁶.

Carbon source in the medium affects growth and survival of any kinds of microbial species. In order to find out carbon source that suits the growth and development of *T. viridae*, 5 Erlenmeyer flasks each containing 100 mL PDB were individually added with 1% of carbon sources, such as cellulose, carboxymethyl cellulose, glucose, sucrose, and maltose respectively. Five mL (10^8 conidia mL⁻¹) inoculum was added in each of these flasks followed by incubation at 40°C for 6 days and growth in terms of biomass (dry weight) was measured¹⁷.

Having optimized all parameters at shake flask level, the process was scaled up to 100 L capacity fermenter. A 100 L capacity fermenter containing 50 L of sterile PDB was inoculated with 500 mL of inoculums of *T. viridae* and incubated at 40°C under controlled conditions for days 10 at 100 rpm. Followed by homogenization of broth in a blender and mixing of *T. viridae* with (1:2 ratio), to this mixture, carboxy methyl cellulose was added as sticker solution. This mixture was allowed to dry under shade leaving 30% moisture. It was aseptically stored in white propylene bags for 6 months under laboratory conditions at 28°C¹⁷.

T. viridae was mixed with charcoal powder, talc, neem cake, gypsum, and soybean oil (1:2 ratio), carboxymethyl cellulose was added as a sticker. This mixture was stored at 28°C and viability of *T. viridae* was checked after every 30 days up to 200 days¹⁶. Total viable count of the fungus was taken as a measure of survival using the following formula.

$$\text{CFU} = \frac{\text{Average No. of colonies}}{\text{Weight of sample}} \times \text{dilution factor}$$

In order to confirm the antifungal and biocontrol potential of *T. viridae*, the *in vitro* antifungal assay was conducted against phytopathogenic fungi namely *R. solani* and *F. oxysporum* using poisoned food technique¹⁸. For this purpose, 10 days uniformly grown *T. viridae* was allowed to filter and the aliquots

(2 mL) of the strain were placed in sterile Petri plate followed by adding 25 mL of PDA. This was followed by growing *R. solani* and *F. oxysporum* at center in the form of 5 mm disc at 28°C for 6 days and growth inhibition (reduction) of both phytopathogenic fungi was recorded.

Results

Effect of temperature on growth of *T. viridae*

Temperature is an important factor affecting growth and activity of all microorganisms. Both temperatures (low and high) affected growth and sporulation in *T. viridae*, temperature of 5, 20 and 25°C; prevented the growth of fungus, while $\geq 30^\circ\text{C}$ supported the growth; optimum growth occurred at 40°C with scanty sporulation. However, further increase in temperature to 45°C affected growth and sporulation (Fig. 1). Singh *et al.*¹⁶ have reported that media, temperature and pH had a profound effect on the growth of fungi and they recorded maximum growth of *T. harzianum* (1.42 g), *T. viride* (1.35 g), *T. asperellum* (1.27 g), *T. longibrachiatum* (1.24 g), *T. atroviride* (1.7 g) and *T. koningii* (1.21 g) at 25°C and found less growth at temperature above 30°C. Singh *et al.*¹⁶ and Zehra *et al.*¹⁹ have also reported optimum growth of *T. harzianum* between 25-40°C. Kim *et al.*²⁰ have claimed that tolerance to high temperature in *Trichoderma* spp. depends on media composition and conidial maturation phase. *Trichoderma* spp. growing over the wide range of temperature offers added advantages for its application in ambient as well as temperate soil.

Effect of pH on growth of *T. viridae*

The pH of the medium is another important factor that affects the growth and activity of the organism.

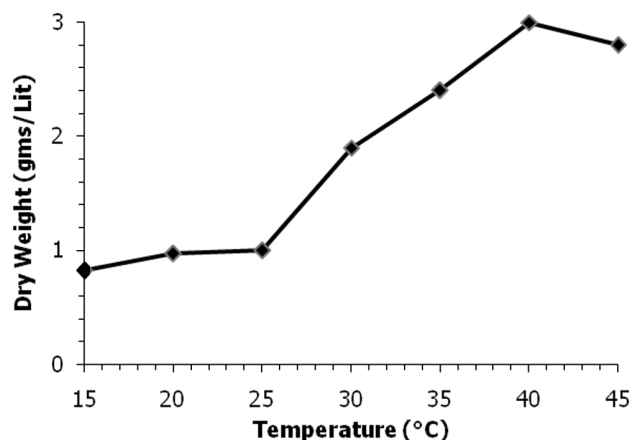


Fig. 1 — Effect of temperature on growth of *Trichoderma viridae*

Maximum growth of *T. viridae* was observed at pH 6.0, while highly acidic and basic pH values affected growth as well as sporulation in *T. viridae*. pH 4.0 supported luxurious growth but it affected sporulation while at pH 8.0 affected growth and sporulation. Ali *et al.*²¹ observed a reduction in colony diameter, growth rate and sporulation in different isolates of *Trichoderma* spp. at pH 8.0 as compared to these characteristics at pH 4.0. Study of Singh *et al.*¹⁶ confirmed that *Trichoderma* spp. grew better in acidic conditions. The acidic ambient pH has a major regulatory effect on biomass production in all *Trichoderma* spp.²¹ hence information on the influence of pH on growth on *T. viridae* is prerequisite for its application in agricultural soil that differs in pH²². The growth of *Trichoderma* spp. is more efficient in acidic than alkaline soils and they modify the rhizosphere soil by acidifying the soil. pH of media regulates mineral availability and affects enzyme activity and thus growth and metabolism of fungus.

Effect of carbon source on the growth of *T. viridae*

Among the different carbon sources used for cultivation of *T. viridae*, optimum (1.25 g) growth (biomass) was recorded in PDB amended with cellulose followed by carboxymethyl cellulose (1.20 g), glucose (1.04 g) while sucrose and maltose-containing PDB produced lowest (0.5 g) biomass. Utilization of cellulose can be attributed to cellulase producing ability of *T. viridae*^{14,23}. We report maximum biomass of *T. viridae* in cellulose as renewable, abundantly available, cost-effective and best utilizable carbon source *vis-a-vis* sucrose as reported by Rajput *et al.*¹⁷. Use of cellulose or cellulosic waste as substrate offers great advantages of being eco-friendly and cost effective for cheaper production of *T. viridae*.

Determination of shelf life of *T. viridae* in various carriers

At initial phases, CFU count was high and declined gradually during storage conditions in all preparations (Table 1). Neem cake was found as an excellent carrier for *T. viridae*, considerably slow reduction in CFU count from an initial count of 60×10^6 cfu g⁻¹ to 35.78×10^6 cfu g⁻¹ occurred after prolonged storage 200 days at 28°C. While soybean oil and gypsum gave 43.78×10^6 cfu g⁻¹ and 32.78×10^6 cfu g⁻¹ after 30 days storage which was further reduced to 16.79×10^6 and 10.00×10^6 cfu g⁻¹ respectively after 60 days storage indicating their inefficiencies in keeping the strain viable for a longer duration. Earlier researchers have found compatibility of *Trichoderma* spp. with

Table 1—The shelf life of *Trichoderma viridae* in different carriers

Carrier	CFU 1×10^6						
	30 days	60 days	90 days	120 days	150 days	180 days	200 days
Charcoal powder	59.45	59.35	53.23	46.34	41.67	38.46	34.69
Talc	59.00	59.00	52.56	45.23	40.39	35.68	30.89
Neem cake	60.00	60.00	52.54	47.23	42.36	40.78	35.78
Gypsum	32.78	32.78	29.78	23.98	18.75	12.88	10.00
Soybean oil	43.78	43.76	39.78	30.56	25.79	20.35	16.79

sorghum²⁷ and other carrier materials. Rajput *et al.*¹⁷ reported shelf life of 60-75 days on different substrates. TVC and shelf life of *T. viridae* were dependent upon the compatibility of strain and nutritional status of substrate and media components²³. We report neem cake as a natural, abundantly available, cost-effective and renewable carrier that also gives longer shelf life *vis-a-vis* present day used synthetic carriers that possess shorter shelf life, non-renewable, unsuitable and costly.

Evaluation of the antifungal potential of *T. viridae*

In vitro inhibition of growth of *R. solani* and *F. oxysporum* was observed in plates seeded with *T. viridae*. A 100% inhibition of *R. solani* and *F. oxysporum* growth was evident in test culture; *T. viridae* completely inhibited the mycelial growth and sporulation of these fungal pathogens. Antifungal activity of *T. viridae* is attributed to its ability to produce a variety of extracellular hydrolytic enzymes, non-volatile compounds, and antifungal antibiotics²⁴⁻²⁵. Darwin *et al.*²⁶ claimed antifungal activity of *T. viridae* against fungal pathogens. Saravankumar *et al.*²⁷ also reported antagonistic activity of *T. harzianum* against *Fusarium* spp. and observed a 86% reduction in the occurrence of disease due to the application of *T. harzianum*. Zehra *et al.*¹⁹ and Ali *et al.*²¹ have reported antagonistic activity of cellulase and chitinase producing *T. viridae* against *F. oxysporum* and *Alternaria alternate*. Many researchers reported antifungal activity of *T. asperellum* under *in vitro* and green house conditions and claimed 67-86% inhibition in growth of fungal pathogens²⁸⁻²⁹. We report complete (100%) inhibition of growth and sporulation in *R. solani* and *F. oxysporum*.

Conclusion

From the results of our above study, we can conclude that neem cake to be an effective carrier for the viability of *Trichoderma viridae* under natural soil condition. The growth of fungus under highly acidic

condition and at a higher temperature (40°C), can be useful for its applications in acidic soil and temperate climate. Utilization of cellulose as carbon substrate and good shelf life in neem can make fungus as a more sustainable, eco-friendly and cost-effective biocontrol agent and plant growth promoting fungus.

Conflict of Interest

The authors declare no conflict of interest.

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