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Mass Production of *Trichoderma viride* with Locally Available Materials

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Abstract Mass production of *Trichoderma viride* (*T. viride*) was done using 11 locally available cheaper substrates. Among the 11 substrates tested for the growth of *T. viride*, maximum growth was observed on rice bran-cowdung-jaggery (T_4) where the mean population (x 10^8 cfu/g) at 15, 30, 45, 60, 75 and 90 days were 42.50x 10^8 cfu/g, 113.33x 10^8 cfu/g, 293.33 × 10^8 cfu/g, 165.8 × 10^8 cfu/g, 62.5 × 10^8 cfu/g and 15.83 × 10^8 cfu/g, respectively. Lowest population was observed on rice bran-mustard cake-sugarcane juice (T_7) where the mean population were 7.50 × 10^8 cfu/g, 38.33 × 10^8 cfu/g, 115 ×10^8 cfu/g, 55 × 10^8 cfu/g, 25 × 10^8 cfu/g and 3.33 ×10^8 cfu/g, respectively. The population of *T. viride* under direct microscopy observation (with the help of haemocytometer) was also found to be higher in rice bran-cowdung-jaggery (T_4). Room temperature showed significant effect on the mass production of *T. viride* and maximum population of 226.6 × 10^8 cfu/g was obtained in rice bran-cowdung-jaggery (T_4) at 45 days (28°C, 80% RH) and the lowest population 103.33 × 10^8 cfu/g was obtained in rice bran-mustard cake-sugarcane juice (T_7). Under direct microscopy observation, maximum population was also observed in rice bran-cowdung-jaggery (T_4). Growth of *T. viride* was found to be best during May to October (26–30°C, 80–95% RH) in all the 3 selected substrates tested viz., rice bran-cowdung-jaggery (T_4), Rice bran-jaggery (T_5) and rice bran (T_6).

Keywords Mass production, *Trichoderma viride*, BOD, Room temperature, Substrates.

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Introduction

The fungus *Trichoderma* was described as early as 1794 by the mycologist Persoon [1]. In the recent years, control of plant pathogens using microbial bioinoculants has been considered as a potential control strategy due to the environmental pollution caused by excessive use of chemical pesticides. Biological control of pathogens using *Trichoderma viride* is very promising method against soil borne plant parasitic

fungi [2, 3]. Therefore, considering the cost of chemical pesticides and hazardous involves, biological control of plant diseases appears to be an effective and ecofriendly approach being practice worldwide [4]. Further biological control strategy is highly compatible with sustainable agriculture and has a major role to play as a component of integrated pest management (IPM) program [5]. Large scale production, along with shelf life and establishment of bioagents in targeted niche, determine the success of biological control [6]. Development of acceptable easily prepared and cost effective formulations for delivery should be major goal. For mass multiplication of bioagent through solid state fermentation technology an enormous quantity of spore biomass is needed. Various substrates like sugarcane baggase, fruit juice waste, vegetable waste, rotten wheat grains are being used for mass multiplication of *Trichoderma viride* with various degree of success [7, 5]. Moreover a huge amount of solid waste like sugarcane baggase, fruit juice wastes, vegetable waste and rotten wheat grains increases pollution and disposal problems [8]. However, a comparative study to find the best and cheap substrate is lacking. Therefore, looking towards need for large scale cost effective production of ecofriendly biopesticide, present investigation was carried out to evaluate locally available cheaper substrates for mass multiplication of *Trichoderma viride*.

Materials and Methods

Preparation of substrates

Different substrates were studied *in vitro* for the mass production of *T. viride*, where rice bran was kept common for each substrate mixture viz., Rice bran + Mustard cake + Jaggery (T_1), Rice bran + Saw dust + Jaggery (T_2), Rice bran + Crushed charcoal + Jaggery (T_3), Rice bran + Cowdung + Jaggery (T_4), Rice bran + Jaggery (T_5), Rice bran alone (T_6), Rice bean + Mustard cake = Sugarcane juice (T_7), Rice bran + Saw dust + Sugarcane juice (T_8), Rice bran + Crushed charcoal + Sugarcane juice (T_9), Rice bran + Cowdung + Sugarcane juice (T_{10}) and Rice bran + Sugarcane juice (T_{11}). The substrates mixture were moistened and 200 g of substrates was filled into the double layered polypropylene bag (20 cm × 18 cm), plugged with cotton balls and tied with fine thread and finally sterilized in autoclave at 1.4 kg/cm^2 for 30 minutes for two consecutive days. Each substrate was tested under two conditions viz., room temperature and BOD (March 2013 to May 2013).

Preparation of mycelia form of inoculums of *Trichoderma viride*

Trichoderma viride was collected from the Department of Plant Pathology, College of Agriculture, Central Agricultural University. For mycelial preparation of *T. viride*, mycelia disc of 5 mm diameter from young growing region of four days old culture of *T. viride* was taken and inoculated into Erlenmeyer flasks (100 ml) containing 50 ml potato dextrose btoth medium. Inoculated flasks were incubated at 28 ± 1°C for 10 days inside BOD incubator. When the medium was fully covered with the *Trichoderma*, mycelial mat was harvested by passing through Whatman No. 42 filter paper and homogenized with a stirrer. The mycelial suspension (1 × 10^5/ml conidia) was prepared and confirmed by haemocytometer and inoculated into polypropylene bags containing different substrate mixtures and incubated at 28 ± 1°C inside the BOD for 15 days with periodical shaking to avoid formation of clump and to enhance uniform growth and sporulation of *Trichoderma* in the medium. At the same time all the treatments were incubated at room temperature separately with three replications for each treatment.

Cultural characteristics

Visual observations on the fungal growth like spore formation, sporulation, changes in color and area coverage by mycelium in different substrates were recorded at 15 days interval upto 90 days.

Estimation of population of *Trichoderma viride*

For population estimation and spores viability of each treatment, observations were done at 15 days interval till 90 days of inoculation, using two methods viz.,

Serial dilution plate technique

In this method, *Trichoderma* culture were collected with the help of spatual from the substrate mixture and crushed to get smaller particles. One gram of substrate was suspended in 250 ml Erlenmeyer flasks of 100 ml sterilized distilled water. Samples were shaked for 20–30 minutes on a rotary shaker at 250 rpm and dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were made for each substrates. An aliquot of 0.1 ml of substrate suspension was dispensed on *Trichoderma*- selective media (TSM). The plates were incubated at 28 ± 1°C for 24 h. Three replications were kept for each observation. Population was counted the next day with the help of colony counter. Observations were taken at 15 days interval viz., 15, 30, 45, 60, 75 and 90 days after inoculation. The total number of *Trichoderma* in colony forming unit per gram (cfu/g) were calculated.

Direct microscopy method

Samples prepared as in serial dilution plate method were used for direct microscopy count of *Trichoderma*. One mililiter of substrate suspension was transferred with the help of micro pipette into haemacytometer and observed under the compound microscope. Also observation was taken under binocular microscope at 40x with phase contrast (AHN Germany made, model premium) with image analysis (Biowizard version 4.2). Number of spores, mycelia fragments and chlamydospores were counted and calculated by following formula:

$$\text{Spores/ml} = \text{Average number of spores} \times (2.5 \times 10^4) \times (\text{Dilution factor})$$

At least five observations were recorded while counting the number of spores, mycelial fragments and chlamydospores of *T. viride*.

Effect of room temperature on the mass production of *Trichoderma viride*

Effect of room temperature on the production of *T. viride* was studied and temperature was recorded every day (March 2013 to May 2013) by using Hygrometer (Thermo). Effect of temperature on *Trichoderma* mycelia growth and spore production were observed and recorded at 15 days interval upto 90 days of inoculation as mentioned above (Serial dilution plate and direct microscopy method).

Effect of the months on the mass production of *Trichoderma viride*

The effect of different months viz., April 2013, May 2013, June 2013, July 2013, August 2013, September 2013, October 2013, November 2013, December 2013, January 2014, February 2014 and March 2014 on the mass production of *T. viride* was studied in the laboratory. The best three substrates were selected and studied for the best month suitable for maximum production of *T. viride* was kept inside the room temperature for one month and population count were made by serial dilution plate and direct microscopy method. Test was made every month for one year. Three replications were kept for each treatment. Temperature and relative humidity were recorded everyday for the whole year with the help of Hygrometer (Thermo).

Results and Discussion

Cultivar characteristics of *Trichoderma viride* in different substrates

The growth of *T. viride* in different substrates except in charcoal was observed to produce white/off white mycelium at first, which gradually changed to yellowish green color. The yellowish green color was more prominent at later stage and further with increased in duration, mycelium spread over the surface of substrates which becomes darker in color due to abundant sporulation. In charcoal substrate, the growth was while initially and changed to black in later stage. These observations are in conformity with the findings of Sobita and Anamika [9] who observed that *T. viride* produced hyaline color at first, which gradially changed to yellowish green color in latter stage, yellowish green color was more prominent at later stage

and become darker in color due to abundant sporulation . Similar, observations were also found by Rini and Sulochana [10], white mycelia growth of *Trichoderma* was observed on sorghum grains on the third day of incubation and later it covered the entire surface of the substrate with profuse green sporulation. In the initial stages spreading of the bioagent in substrates was fast because of abundant of nutrients and later stages, nutrients exhausted as a result growth was ceased and started reducing.

Estimation of population of *Trichoderma viride*

The results of the experiment showed that mean population was found to increase steadily up to 45 days and there after it declined. The population of the *T. viride* under BOD condition at 15 days of incubation (DOI) ranged from 7.50×10^8 cfu/g to 42.50×10^8 cfu/g, at 30 days of incubation ranged from 38.33×10^8 cfu/g to 113.33×10^8 cfu/g, at 45 DOI ranged from 115×10^8 cfu/g to 293.33×10^8 cfu/g, at 60 days of incubation ranged from 55×10^8 cfu/g to 165.8×10^8 cfu/g, at 75 days of incubation ranged from 25×10^8 cfu/g to 62.5×10^8 cfu/g and at 90 days of incubation ranged from 3.33×10^8 cfu/g to 15.83×10^8 cfu/g where the maximum population (42.50×10^8 cfu/g, 113.33×10^8 cfu/g, 293.33×10^8 cfu/g, 165.8×10^8 cfu/g, 62.5×10^8 cfu/g and 15.83×10^8 cfu/g) was recorded with rice bran-cowdung-jaggery and the lowest population (7.50×10^8 cfu/g, 38.33×10^8 cfu/g, 115×10^8 cfu/g, 55×10^8 cfu/g, 25×10^8 cfu/g and 3.33×10^8 cfu/g) was recorded in rice bran-mustard cake-sugarcane juice. It was also observed that the population of *T. viride* when observed directly under microscope with the help of haemocytometer was found to be higher than when grew under *Trichoderma*- selective media (TSM).

Increase in the rate of population upto certain period and reduction later may be the result of decrease in the amount of nutrient available in the sustrates. Under microscope all the spores, mycelia fragments and chlamydospores both viable and non viable were counted, whereas when grew under *Trichoderma*-selective media only the viable ones will germinate and that might be the reason for higher population under microscope than when grew under *Trichoderma*-selective media (TSM). Several workers supported these results, Palanna et al. [11] found that the treatment FYM + goat manure extensively supported the growth of *T. viride* and recorded the maximum growth 571.38 mg mycelial dry weight. Rini and Sulochana [10] reported that cowdung-neem cake-jaggery (3%) maintained maximum inoculums density on the 10^{th} day and that locally available organic media viz., rice bran, cowdung and neem cake are excellent sources of nutrition for antagonistic fungi like *T. harzianum* and *T. viride*. Bheemaraya et al. [12] used various agrowaste materials for mass multiplication of *T. viride*, *T. harzianum* and *T. piluliferum* and reported that rice husk was found to be superior substrate [12]. Chaudhari et al. [5] reported higher spore and mycelia on sugarcane baggage (91.3×10^8/g) and found that organic waste, vegetable waste and fruit juice to be cheapest and best suitable media for the large-scale production of *Trichoderma* spp. Yadav [13] screened substrates for mass multiplication of *T. viride* and *T. harzianum* reported that rice husk, wheat bran, maize husk and saw dust were the most suitable substrate. Kumar and Palakshappa [14] reported that maximum population was obtained in sterilized local cowdung.

Effect of room temperatures on the mass production of *Trichoderma viride*

Results indicated that the population of the *T. viride* under room temperature condition at 15 dats (20°C, 72% RH) ranged from 5.83 to 38.33×10^8 cfu/g, at 30 days (26°C, 86% RH) ranged from 36.67 to 82.5×10^8 cfu/g, at 45 days (28°C, 80% RH) ranged from 103.33 to 226.6×10^8 cfu/g, at 60 days (31°C, 76% RH) ranged from 47.5 to 130.83×10^8 cfu/g, at 75 days (28°C, 80% RH) ranged from 16.67 to 41.67×10^8 cfu/g and at 90 days (35°C, 71% RH) ranged from 2.5 to 13.33×10^8 cfu/g where the maximum population (38.33×10^8 cfu/g, 82.5×10^8 cfu/g, 226.6×10^8 cfu/g, 130.83×10^8 cfu/g, 41.67×10^8 cfu/g and 13.33×10^8 cfu/g) were recorded with rice bran-cowdung-jaggery which was similar to the findings under BOD condition. In comparison to the population under BOD and room temperature condition, population was higher in BOD. In

BOD the temperature was fixed (28 ± 1°C) which is the optimum temperature for growth [15], whereas in room temperature there was fluctuation in temperature (20°C to 35°C) and relative humidity (71% to 81%) and this may be the reason for low population in room temperature compared to BOD.

Effect of different months on the mass production of *Trichoderma viride*

The effect of different months in the selected best three treatments viz., T_4 (rice bran-cowdung-jaggery), T_5 (rice bran-jaggery) and T_6 (rice bran) significantly higher CFU, spore and mycelial fragments was obtained during the month of May-October (26–30°C, 80–95% RH). Although in T_4 higher CFU count (86×10^8 cfu/g) and spore (95.5×10^8/g) were obtained during the month of September (28°C, 91% RH) and higher amount of mycelia fragments (8.5×10^8/g) in the month of May (26°C, 89% RH). Whereas, in T_5 high CFU count ($73.5–75 \times 10^8$ cfu/g) was obtained during September–October (26-28°C, 91–93% RH), maximum number of spores ($73.5–77 \times 10^8$) was recorded in May and September (27–29°C, 85–91% RH) and highest amount of mycelial fragments (12.5×10^8/g) in the month of October (26°C, 93% RH). And in T_6 high CFU count ($73–73.5 \times 10^8$ cfu/g) was during September–October (26–28°C, 91–93% RH); and maximum number of spores ($67.5–70.5 \times 10^8$) in May and September–October (26–29°C, 85–93% RH). These findings are in consistent with the findings of Al-Fattah who reported that maximum growth of *T. viride* occured from 25°C to 30°C and decreased at 35°C [16]. The potimum temperature for growth of *Trichoderma* spp. was obtained at 28°C and high relative humidity [17]. Sargin et at. [15] found that deviation from 28°C, which is optimum for most of the fungi, resulted in the reduction of micropropagule counts. Zuriash and Tesfaye [18] evaluated agro-industrial wastes for conidial production of *Trichoderma* isolates under solid state fermentation and reported that wheat straw, vegetable wastes and tea waste supported good conidia production of *Trichoderma* isolates and were better substrates for conidia production from economic and environmental point of view. Thus, mass-scale production of *Trichoderma* spp. would have great potential for commercial use. However, the cost of the raw materials is one of the major limitations behind the restricted commercial production and widespread use of biocontrol. To overcome the cost limitation, the present study has given informations regarding the easily available and cheap raw materials for mass multiplication of *T. viride.*

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