Research Article

Effect of Different Growth Media and Physical Factors on Biomass Production of *Trichoderma Viride*

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ABSTRACT

In the past decade, excessive use of chemical pesticides has increased environmental contamination which in turn increased the importance for biopesticides to control different plant pathogens and diseases. *Trichoderma viride* is potent biocontrol agent. To obtain the favourable conditions for the biomass production of *Trichoderma viride*, the present study was conducted on different growth media and various physical factors i.e. pH, temperature and humidity. The best growth of *Trichoderma viride* was observed on Sabouraud Malt Yeast extract Agar (SYMA) medium with colony diameter of 2 cm after 5 days of incubation. The stationary phase of growth of *Trichoderma viride* occurred from 9th day with the decline phase starting from the 11 day of incubation. *Trichoderma viride* favored acidic while poor mycelia development was observed on alkaline medium. Moreover, alkaline pH had inhibitory effect on the growth and development of mycelia and pigmentation of the fungus. *Trichoderma viride* could grow at a wide range of temperature between 20-30ºC. Best growth and sporulation of the fungus was observed at 28ºC, colony diameter of 2.6 cm and optimum pH at 6 with 3.7cm colony diameter and optimum relative humidity for sporulation was obtained at 80-90%.

KEY WORDS: biomass production, mycelial growth, pH, relative humidity, sporulation, temperature, *trichoderma viride*

INTRODUCTION:

The most widely used control measure for suppressing several soil borne fungal pathogens diseases are the use of fungicides. However, problems encountered, such as development of pathogen resistance to fungicides, various insecticides and inability of these to protect the roots of mature plants has its own limitations. The chemical methods to control soil borne diseases are very difficult and costly. Considering these limitations, bioagents are important and inexpensive in this direction.

Biological control is an eco-friendly approach towards plant protection where specific micro-organisms interfere in host. Antagonistic biocontrol agent is a potential approach for managing plant diseases. *Trichoderma viride* is one of the bioagents against several fungal pathogens which possess better ability to promote plant growth and soil remediation activity compared to their counterparts (virus, bacteria, nematodes and protozoa).[1] These antagonistic fungi are most common among fungal biocontrol agents because of their multiple characteristics, namely antagonism and plant-growth stimulation. Thus, mass-scale production of *Trichoderma* spp. would have great potential for commercial use.

Fungi being saprophytic or parasitic require an external source of nutrition. External source of carbon (C), hydrogen (H), oxygen (oxygen) and nitrogen (N) are utilized by *Trichoderma* sp. For the production of *Trichoderma* spp, addition of glucose, cellulose, soluble starch and molasses is the conventional method for semi-synthetic media.[2,3,4,5]

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The first of three principle elements are provided by carbohydrates, while nitrogen source may be in organic or inorganic form. Among different sources of carbohydrates, glucose is the preferred one for nearly every fungus. The enzyme system of fungi is well developed to synthesize other essential requirements from these basic elements.[6] *Trichoderma viride* is a potent organism serving from decades in biological control because it decreases or eliminates allelopathic chemicals, prevents the allelopathic effects of Vesicular-Arbuscular Mycorrhiza (VAM) fungi, increase acidification of soil thereby chances of pathogenic fungi are reduced due to antagonistc interactions and inhibits the growth of true dry rot fungus *Serpula lacrymans*, which is the most significant fungal cause of damage to timber in built environment in temperate regions of the world. In other case sudden death syndrome (SDS) in soyabean is one of the most destructive disease caused by *Fusarium virguliforme*, has gradually increased importance to discover biocontrol agents.[7,8]

Application of chemical fungicide has been replaced by biocontrol agents because of the emergence of fungicide-resistant strains and public concerns regarding the health and environmental impacts of these chemicals. During the past few decades, several potential biocontrol organisms have been isolated, characterized and commercialized. Now a days, biocontrol of plant diseases has received more consideration in plant disease control.[9,10] Thus, the present study was conducted to test the efficacy of different growth media and various physical factors on biomass production of *Trichoderma* spp. under invitro conditions.

MATERIALS AND METHODS:

Identification: Slide culture technique was adopted for the identification of different isolates of *Trichoderma viride*. Sterilized moist chamber was prepared by keeping thin cotton pad, a wet filter paper and a slide inside a sterilized petriplate. Simultaneously, saboraud dextrose agar medium was prepared and poured on a sterilized glass plate in the form of a thin film. On solidification this film was cut into small cubes with flamed scalpel and was placed on slides inside the moist chamber, later inoculated with fungal spores separately. These slide cultures were incubated at 28°C. After sporulation, the slide was stained with lactophenol cotton blue. For permanent mounts the cultures were stained with DPX.[11,12] Fungal identification was done on the basis of morph taxonomic characteristics whereas, microscopic examinations were carried out to ensure that the cultures were not mixed.[13]

Evaluation of growth and spore production of *Trichoderma viride*: Initially, *Trichoderma viride* cultures were grown on potato dextrose agar (PDA) and incubated at 25º to 30ºC for 5 days. Later, 0.5cm mycelium was transferred from 3 day old culture in 500 ml flasks containing potato dextrose broth (PDB) to obtain pure culture. Homogenous Trichoderma sp. culture was obtained by mixture of 0.1ml spore suspension grown in PDB, containing 10^6 spores/ml in water with 0.05% Tween 80 and spreading evenly on PDA and incubated at 28±1ºC in the dark. After two days, mycelium discs were removed with a sterile cork borer and transferred to different media i.e. Potato dextrose Agar (PDA), Potato Carrot Agar (PCA), Malt Extract Peptone Agar (MEP), Sabouraud Malt Yeast Extract Agar (SMYA), Sabouraud Dextrose Agar (SDA), COON'S Agar, Czapek Dox Agar, Richards Agar and Casein Agar to identify maximum growth of colony. Measurements of the mycelia growth were observed after five days of incubation at 28±1ºC (Table 1). The diameter of the colonies was estimated by calculating the mean of two perpendicular measurements. The sporulation rate was assessed after six days of incubation, individual petridishes were rinsed with 1ml of Tween 80 and the conidia were scraped off carefully. The spore concentration was determined with a haemocytometer and the viability of the conidia was examined on SMYA after 24 hours of incubation at 28±1ºC. [14,15,17]

The physical parameters were studied using SMY broth due to its potentiality as best growth media. Growth pattern of *Trichoderma viride*: 25 ml SMY broth each was inoculated with 1 ml of conidial suspension of *Trichoderma viride* and was incubated at 28 ±1ºC on rotary shaker at 25 RPM for agitation. The weight of mycelia (fresh and dry weight) was taken from 2 to 15 days of incubation. [17]

Determination of pH: 40 ml of SMYA media of pH gradient from 2 to 12 was prepared, sterilized and was poured in sterilized petriplates. After solidification of the medium, plates were inoculated by spot inoculation with *Trichoderma viride*. The inoculated plates were incubated at 28±1ºC. The colony diameter and characteristics were observed daily after an incubation period of 48 hours upto 7 days.

Effect of Temperature: To study temperature effect on growth of *Trichoderma viride*, inoculated petriplates of SMYA media were incubated at different temperature viz, 5, 10, 15, 20, 25, 28, 30, 35 and 40ºC

and observation were recorded after 7 days of incubation [15,16,18].

Effect of Relative Humidity (RH): Relative humidity on the growth and sporulation of *Trichoderma viride* was observed on solidified SMYA media. Inoculated petriplates were incubated at different relative humidites viz, 50, 70, 80, 90, 95 % [18]. Observations were recorded after 7 days of incubation.

RESULTS:

Influence on the growth and sporulation of fungi depends upon many factors *viz.*, Temperature, light, micro and macro chemical compositions of the substrate. In the laboratory conditions, different growth patterns were observed for *Trichoderma viride* and was found capable to grow on almost all the media tested. But, Sabouraud Malt Yeast extract Agar proved to be the best suitable medium for the growth of *Trichoderma viride* with a colony diameter of 2.9 cm after 5 days of incubation amongst other media studied.

Growth is considered as an irreversible increase in the mass/volume of an organism that occurs after a given period of incubation, in nature or in laboratory. Growth of *Trichoderma viride* mycelium was performed using juvenile growth stage from SYM agar. It is necessary to consider vegetative growth in fungi separately as it exhibits different modes or patterns of growth. The results revealed that the stationary phase of growth of *Trichoderma viride* occurred from 9th day with the decline phase starting from the 11th day of incubation (Table 2).

Effect of hydrogen ion concentration or pH on the growth of *Trichoderma viride* favored slight acidic condition where optimum pH was 6, while poor mycelia development was observed on alkaline medium. At alkaline pH, 10, 11 and 12, inhibitory effect was observed on the growth and development of mycelia and pigmentation of the fungus.

In our study, the effect of temperature was observed. The best growth and sporulation of the fungus was obtained at 28ºC and to lesser extent at 30ºC, as exhibited by colony diameter i.e. 2.9 and 2.5cm and sporulation/spore count i.e., 5918 and 5433 respectively. At 25ºC, the colony diameter was 2.6 cm and sporulation was good (5473). Colony characteristics were similar, except that much flosses growth was visible in sporulating colony at 25, 28 and 30ºC. There was no growth below 10ºC, but inoculum started rooting in the media. At 15ºC, greenish mycelial growth was observed with a diameter of 0.6cm. Moreover, at 35ºC the colony was small with a diameter of 0.4cm without sporulation and there was no growth at 40ºC (Table 4).

The present isolate of *Trichoderma viride* was capable to grow and sporulate at different levels of relative humidity. The fungus attained its best growth and sporulation at 80 and 90%RH, with colony diameter measuring 3.0 and 2.7 cm respectively. At 50 and 70% RH, the colony diameter was 1.2 cm with moderate sporulation; at 90% RH and 95% RH, colony diameter was 2.7 and 2.2 cm respectively. The colony characteristics at different humidities were similar except that the fungal growth was highly floccose and powdery in the sporulating fungus at 80%RH with 3.0cm diameter. Thus, optimum relative humidity increased growth at 80% RH, but decreased with increase in %RH at 95, as sporulation decreased with spore count 1.2×10^5 spores/ml. The minimum time required for sporulation in saturated atmosphere was found to be 3 days. *Trichoderma viride* sporulated with relative humidity as low as 50% which proves its superiority over other isolates of *Trichoderma*.

Table 1: Effect of Different Media on the growth of *T. viride.*

S.No	Name of Medium	Colony Diameter (cm)
1	Casein Agar	1.7
2	Coon's	1.0
3	Czapek Dox Agar	1.4
4	Malt Extract Peptone Agar (MEP)	1.4
5	Potato Dextrose Agar (PDA)	0.9
6	Potato Carrot Agar (PCA)	2.0
7	Richard's Agar	1.3
8	Sabouraud Dextrose Agar (SDA)	2.6
9	Sabouraud Malt Yeast extract Agar (SYM)	2.9

DISCUSSION:

Influence on the growth and sporulation of fungi depends upon many factors *viz.*, Temperature, light, micro and macro chemical compositions of the substrate. In the laboratory conditions *Trichoderma viride* was found capable to grow on almost all the media tested with different growth patterns. Sabouraud malt yeast extract agar proved to be the best suitable medium for the growth of *Trichoderma viride*. Moreover, the yeast extract in the medium acted as an inducer for the growth of certain fungi and is responsible for the instant growth radially.[13]

Growth is considered as an irreversible increase in the mass/volume of an organism that occurs after a given period of incubation, in nature or

Table 2: Growth Pattern of *T. viride*

Days of Incubation	Weight of filter paper (g) (A)	Total weight (g) (B)	Wet Mycelial Weight (g) C= B-A	Dry Mycelial weight (g) (D)	Loss in weight = C-D
2	0.546	0.679	0.133	0.061	0.072
3	0.338	0.540	0.202	0.094	0.108
4	0.543	0.788	0.245	0.084	0.161
5	0.407	0.594	0.187	0.067	0.120
6	0.496	0.673	0.177	0.089	0.088
7	0.361	0.636	0.275	0.109	0.166
8	0.414	0.530	0.116	0.052	0.064
9	0.558	0.847	0.289	0.106	0.183
10	0.564	0.752	0.188	0.113	0.075
11	0.667	0.903	0.236	0.171	0.065
12	0.585	0.831	0.246	0.118	0.128
13	0.412	0.573	0.161	0.105	0.056
14	0.502	0.631	0.129	0.078	0.051
15	0.571	0.713	0.142	0.116	0.026

Table 3: Effect of pH on the Growth of *T.viride*.

S.No	pH	Colony Diameter (cm)	Colony Characteristics
1	2	0	No Growth
2	3	0	No Growth
3	4	0	No Growth
4	5	2.4	Light Green colony and white outer circumference
5	6	3.7	Thick greenish colony with cottony white outer circumference and elevation at the centre
6	7	2.6	Depressed growth, greenish at centre elevation.
7	8	2.1	Light green cottony colony
8	9	2.0	Thin green colony
9	10	0.7	Depressed Thin cottony colony
10	11	0	No Growth
11	12	0	No Growth

Table 4: Effect of Different Temperatures on the Growth of *Trichoderma viride*

S.No	pH	Colony Diameter (cm)	Colony Characteristics
1	2	0	No Growth
2	3	0	No Growth
3	4	0	No Growth
4	5	2.4	Light Green colony and white outer circumference
5	6	3.7	Thick greenish colony with cottony white outer circumference and elevation at the centre
6	7	2.6	Depressed growth, greenish at centre elevation.
7	8	2.1	Light green cottony colony
8	9	2.0	Thin green colony
9	10	0.7	Depressed Thin cottony colony
10	11	0	No Growth
11	12	0	No Growth

in laboratory. The results revealed that the stationary phase of growth of Trichoderma viride occurred from 9th day with the decline phase starting from the 11th day of incubation. It is necessary to consider vegetative growth in fungi separately as it exhibits different modes or patterns of growth [19].

It was observed that the growth and development of mycelia and pigmentation of *Trichoderma viride* was favored by slight acidic condition and alkaline pH showed inhibitory effect. It

Table 5: Effect of Different Levels of Relative Humidity on the Growth of *T. viride*

S. No	Percent Relative Humidity	Colony Diameter (cm)	Sporulation/ Spore count/ml	Colony Characteristics
1	50	1.2	Moderate/ 0.85×10^4	Colour is initially greenish and become green, colony white in revere side.
2	70	1.5	Moderate/ 0.85×10^4	Colony is initially greenish and become green, white in reverse side
3	80	3.0	Excellent/ 1.8×10^5	Green and and become greenish and white in reverse side.
4	90	2.7	Excellent/ 1.7×10^5	Green and and become greenish and white in reverse side.
5	95	2.2	Good/ 1.2×10^5	Green colony, white in reverse side.

was considered that change in the pH of a medium alters its composition due to weakly ionized constituents, thus, affecting the physiological effects of dissociated and undissociated species. Most of the fungi grow within the pH range 4-8; whereas, many fungi grow over a wider range, and a few have been reported to have a narrower range. [20] However, in the present study *Trichoderma viride* was best grown at pH 6 which declined with increase in pH

Temperature has been considered for a long time to be one of the important factors affecting the natural activity of parasitic fungi. Much information is available on the effect of temperature on growth of entomogenous fungi in vitro. [21,22] Generally, the limit for growth range between 10-35ºC and the optimal fall between 20 and 30 ºC. Robert and Yendol (1971) and Ferron (1978) described the influence of temperature and relative humidity on muscardian fungi and stated that the rapidity of mycelia development and thereby infection depends on temperature.[23,24] Previously, in vitro effect of temperature had been reported for Beaveria bassiana where limit was 5-35ºC with optimum and growth between 20-30ºC.[21,24,25,26]

In various studies conducted, it was observed that *Trichoderma viride* could grow at a wide range of temperature between 20-35ºC. But our results describes the optimum temperature 28ºC to be best for the growth and sporulation of the fungus

Environmental humidity and the interaction of these factors in the ecological complexes, influence the development of a fungal pathogen [27]. Effect of different levels of relative humidity undoubtedly plays a very important role in sporulation on growth of *Trichoderma viride*. The present study suggested that *Trichoderma viride* was capable to grow and sporulate at different levels of relative humidity where it attained its best growth and sporulation at 80 and 90% RH. However, the optimum relative humidity increased growth at 80% RH, but a decrease was later observed with increase in %RH at 95. Lower humidity levels had some sort of retarding effect on the growth and sporulation. [28,29,30] *Trichoderma viride* sporulated with relative humidity as low as 50% which proves its superiority over other isolates.

CONCLUSION:

In recent years, the fertility of soil is deteriorated due to the prevailing chemical pesticides. Scientist is keen to recover the species which could be used without negative impact on ecology. The research broadens the aspects of *Trichoderma viride* to be utilized as an agro product to offset the pest resistance and provide the alternative and cheap source for the mass production of the specie. *Trichoderma viride* had been previously reported as bio-pesticide. Thus, the current study concludes that the mass production of the *Trichoderma viride* can be increased by standardizing the physical factors. It is also concluded that Sabouraud Malt Yeast extract Agar is the most suitable media for the growth and sporulation of *Trichoderma viride*.

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