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# EFFECT OF PHYSIOLOGICAL PARAMETERS ON MASS PRODUCTION OF *TRICHODERMA* SPECIES

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## A B S T R A C T

Mycophagous fungi show antagonistic potential against plant pathogenic fungi and suggested as a promising biocontrol agent that influences by nutritional and environmental parameters. The present research was conducted with the aim to determine optimal environmental and nutritional parameters range for biomass production of *Trichoderma*. The two species of *Trichoderma* isolated from rhizosphere of citrus, wheat and tomato of different localities of district Sargodha, Punjab, Pakistan and later on effect of temperature, carbon, nitrogen and pH were investigated on three strain of *T. harzianum* HM, HK, HC and one strain of *T. asperellum* TH under *in vitro* conditions. All strains produced statistically sufficient mass production at all temperatures but 25 °C as well as 30 °C were found ideal for mass production of *Trichoderma*. A substantial difference in mass production of *Trichoderma* isolates was recorded at different pH levels includes 4.0, 5.0, 6.0, 7.0 and 8.0. The pH range from 5.0 to 7.0 found to be optimum for all species of *Trichoderma*. All fungal species of *Tricoderma* produced extensive mass production on growth media supplemented with carbon and nitrogen sources such as mannose, glactose, sucrose, lactose and sodium nitrite ($NaNO_3$), potassium nitrate ($KNO_3$), ammonium nitrate (NH4NO$_3$) as well as ammonium nitrite ($NH_4NO_3$) respectively. The optimal mass production was recorded on carbon supplemented growth medium as compared to nitrogen supplemented growth medium. Therefore, *T. harzianum* HK produced maximum biomass production among all isolates of *Trichoderma*.

**Keywords**: *Trichoderma* species, biocontrol, temperature, pH, light, carbon and nitrogen sources.

## INTRODUCTION

*Trichoderma* species are successful antagonist having biocontrol abilities against economically important plant parasitic soil borne pathogens and present abundantly in almost all types of soils (Olabiyi and Ruocco, 2013; Kushwaha *et al*., 2014; Shahid and Srivastava, 2014). Biocontrol antagonists play an important role in the management of plant diseases (Hajieghrari *et al*., 2008; Alwathnani and Perveen, 2012; Li *et al*., 2013). Generally, commercial production of *Trichoderma* largely depends upon bulk conidia production that reproduces asexually (Verma *et al*., 2007; Singh and Nautiyal, 2012). Mass production of *Trichoderma* conidia relies on physical and environmental parameters that promote conidiation for commercial production of *Trichoderma* species (Harman *et al*., 2010; Singh *et al*., 2014). *Trichoderma* species have much importance as biological control agents against plant pathogens (Monte, 2001). The environmental factors includes temperature and pH influences mass production as well as mycelial growth of Trichoderma and plant pathogenic fungi (Cavalcante *et al*., 2008; Wang *et al*., 2014). Among these parameters, pH is most imperative factor that affects the mycoparasitic activities of Trichoderma (Kredics *et al*., 2004). A specific and optimum value of physiological and environmental parameters is necessary for maximum growth of biocontrol agent where it can multiply and control the pathogen easily (Hajieghrari *et al*., 2008). Most of the studies on varying pH revealed that sporulation and optimum growth rate of Trichoderma ranging from 2.5 to 7.5 (Bandyopadhyay *et al*., 2003; Begoude *et al*., 2007). *Trichoderma* strains

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are able to grow in varying pH levels with maximum growth rate at 4 pH but the optimum range for rapid growth varies from 4-7 pH (Begoude *et al*., 2007). So, there is need to modify *Trichoderma* strains genetically for acidic and saline soils where there performance can produce better results for reducing the severity level of plant pathogens. Similarly, temperature is also imperative parameter for growth and sporulation of *Trichoderma* species (Puyam *et al*., 2013). Significant information on nutrition of *Trichoderma* are available in literature but very little is well-known about specific carbon and nitrogen nutrients on mass production of *Trichoderma* antagonists (Rajput *et al*., 2014). Therefore, present research work conducted to study effect of different physiological parameters on mass production of Trichoderma isolates.

## MATERIAL AND METHODS

**Isolation and identification of *Trichoderma:*** All isolates were collected from rhizosphere of citrus, wheat and tomato of different localities of district Sargodha, Punjab, Pakistan. Isolation was done from different soil samples on potato dextrose agar (PDA) media followed by soil dilution method.

***Trichoderma* inoculums preparation:** Three strains of *T. harzianum* HM, HK, HC and one strain of *T. asperellum* TH were investigated on potato dextrose agar medium (potato starch 20g; dextrose 20g; agar 20g; distilled water 1000ml) for biomass production (Mustafa *et al*., 2009). Spore suspensions of *Trichoderma* were prepared from 7 days mature fungal colony by addition of distilled water (15 ml) to extricate spore from mycelial growth. The spore concentration (110 spores/ml) obtained by counting of spores with help of heamocytometer to inoculate 100 ml TSM broth in 500 ml flask. The cultures were incubated at 25±2°C in an arbitrary shaker operating at 150 rpm for 2 days. The resultant cultures were washed aseptically with distilled water to eliminate remaining media and then used for further experiments as standard inoculum. A total 10% (v/v) of standard inoculum was inoculated and experiment performed in triplicate. The biomass production was indication of *Trichoderma* growth and mycelial dry weight calculated by using oven dry method.

**Effect of temperature:** The species of Trichoderma were analyzed on various temperatures in order to determine optimum temperature for mass production on potato dextrose agar medium. The conical flasks contain TSM incubated at 20, 25, 30, 35 and 40°C in a shaker operating at 150 rpm for 7 days. The optimum temperature that was supported maximum biomass production used for further subsequent steps.

**Effect of carbon source:** The carbon sources used in this study were mannose, glactose, sucrose and lactose. The nutrients effects on mass production were evaluated in CzepekDox Liquid Medium and medium amended with glycine (1g/L) or glucose (10/g/L) and media were sterilized for 15 minutes at 15 psi, penicillin @100,000 units/lit. and streptomycine @ 0.2 g/lit. were added in media just before pouring to inhibit the bacterial growth (Rajput *et al*., 2015). Carbon sources were filtered sterilized and put into autoclaved media at 20g/L. Each conical flask of 250 ml contains 100ml media inoculated with *Trichoderma* mycelial 5 mm agar plug from 7 days mature fungal colony. Each treatment replicated three times and mass production of Trichoderma species was recorded after 14 days of incubation at 25±2°C.

**Effect of nitrogen source:** The nitrogen sources used in this study were sodium nitrite ($NaNO_3$), potassium nitrate ($KNO_3$), ammonium nitrate ($NH4NO_3$) and ammonium nitrite ($NH_4NO_3$). All remaining procedures were followed as performed in carbon sources but amount of nitrogen sources as basal medium used 2g/L as compared to nitrogen sources.

**Effect of pH:** Isolates of *Trichoderma* species were investigated *in-vitro* at pH 4, 5, 6, 7 and 8. A standard inoculum was inoculated in 100 ml TSM broth into a 500 ml conical flask. Inoculated flask contains TSM growth medium incubated at 25±2°C in an arbitrary shaker operating at 150 rpm for 7 days. The optimum pH that was supported maximum biomass production used for further subsequent steps.

## STATISTICAL ANALYSIS

The readings of experimental results were obtained and data analyzed with the help of statistical software R by using Least Significant Test (LSD). The results which have P<0.05 were considered as significant.

## RESULTS

**Isolation and identification of *Trichoderma:*** All four strains of *Trichoderma* selected among 15 isolates on the basis of morphological characteristics such as length, width of spores and colonies patterns in PDA plates at room temperature such as ranges from light green to dark green according to the description of Kannangara *et al*., 2017.

**Effect of temperature:** All *Trichoderma* species produced good amount of biomass at varying temperatures. *T. harzianum* HK produced maximum biomass (1461 mg) at incubation of 25°C as compared to 30°C and 20°C that results 1396 mg and 1324 mg biomass production respectively. The subsequent maximum biomass produced by *T. harzianum* HM, *T. asperellum* TH and *T. harzianum* HC at 25°C was 1443 mg, 1221 mg and 1158 mg respectively. Biomass production of *T. harzianum* HK was high on all temperature as compared to others *Trichoderma* species. The statistical analysis revealed that there is no significant variation between 25 °C and 30 °C shown in Table 1.

Table 1. Effect of temperature on biomass production of *Trichoderma* species

| Temperature | *T. asperellum TH* | *T. harzianum* HM | *T. harzianum* HK | *T. harzianum* HC | Mean±S.E |
|---|---|---|---|---|---|
| | Mean±S.E | Mean±S.E | Mean±S.E | Mean±S.E | |
| 20 °C | 1159±1.41 $^{b}$ | 1293±1.24 $^{c}$ | 1324±1.00 $^{c}$ | 1049±1.69 $^{c}$ | 1206.7±1. 3$^{C}$ |
| 25 °C | 1216±1.34 $^{a}$ | 1443±1.65 $^{a}$ | 1461±0.90 $^{a}$ | 1158±1.15 $^{a}$ | 1319.9±1.2 $^{A}$ |
| 30 °C | 1220±1.23 $^{a}$ | 1369±1.75 $^{b}$ | 1396±1.23 $^{b}$ | 1100±1.53 $^{b}$ | 1271.7±1.4 $^{B}$ |
| 35 °C | 901±1.98 $^{c}$ | 1129±1.12 $^{d}$ | 1171±1.00 $^{d}$ | 782±1.8 $^{d}$ | 996.2±1.4 $^{D}$ |
| 40 °C | 583±2.36 $^{d}$ | 647±1.36 $^{e}$ | 700±1.20 $^{e}$ | 509±1.16 $^{e}$ | 610.0±1.5 $^{E}$ |
| Mean | 1016.4±1.64 $^{C}$ | 1176.5±1.42$^{B}$ | 1210.8±1.06$^{A}$ | 919.5±1.46$^{D}$ | |

*Small letters are for comparison within column comparison, Capital letters for comparison for comparison among temperature mean, Bold and capital letters are for comparison among species.

**Effect of carbon source:** *Trichoderma* biomass in different carbon amended media was significantly superior than nitrogen amended nutrition. Mannose is best carbon nutritional medium for *Trichoderma* among the carbon sources followed by glactose, sucrose and lactose. All species of *Trichoderma* produced dark green and yellow greenish conidia with prominent concentric rings on lactose, sucrose and mannose respectively. *Trichoderma harzianum* HK biomass production was maximum (1627.2 mg) among *Trichoderma* strains on carbon sources followed by *T. harzianum* HM (1571.0 mg), *T. asperellum* TH (1505.6 mg) and *T. harzianum* HC (1427.1 mg) while all *Trichoderma* strains produced maximum biomass on mannose (2604.5 mg) followed by glactose (2310.2 mg), sucrose (1967.8 mg) and lactose (440.1 mg). The statistical analysis revealed clear differences among *Trichoderma* biomass production on different carbons sources in Table 2.

Table 2. Effect of carbon sources on biomass production of *Trichoderma* species

| Carbon Sources | *T. asperellum TH* | *T. harzianum*HM | *T. harzianum*HK | *T. harzianum*HC | *Mean±S.E* |
|---|---|---|---|---|---|
| | Mean±S.E | Mean±S.E | Mean±S.E | Mean±S.E | |
| Control | 305.8±0.88 $^{e}$ | 375.0±.97 $^{e}$ | 432.4±1.23 $^{e}$ | 247.1±1.51 $^{e}$ | 340.1±1.14 $^{E}$ |
| Glactose | 2274.6±1.17 $^{b}$ | 2329.0±1.21 $^{b}$ | 2425±1.34 $^{b}$ | 2211.8±1.27 $^{b}$ | 2310.2±1.29 $^{B}$ |
| Lactose | 435.6±0.89 $^{d}$ | 500.4±.78 $^{d}$ | 452±1.06 $^{d}$ | 371.6±1.46 $^{d}$ | 440.1±1.04 $^{D}$ |
| Mannose | 2571.9±1.09 $^{a}$ | 2638.2±1.14 $^{a}$ | 2709±1.31 $^{a}$ | 2502±1.52 $^{a}$ | 2605.4±1.26 $^{A}$ |
| Sucrose | 1940.0±1.33 $^{c}$ | 2012.6±1.29 $^{c}$ | 2115±1.17 $^{c}$ | 1802±1.86 $^{c}$ | 1967.8±1.41 $^{C}$ |
| Mean±S.E | 1505.6±1.07$^{C}$ | 1571.0±1.07$^{B}$ | 1627.2±1.2$^{A}$ | 1427.1±1.52$^{D}$ | |

*Small letters are for comparison within column comparison, Capital letters for comparison among carbon sources mean, Bold and capital letters are for comparison among species.

**Effect of nitrogen source:** *Trichoderma* biomass in different nitrogen amended media was significantly lower than carbon amended nutrition. Sodium nitrite ($NaNO_2$) is statistically best nitrogen nutritional medium for *Trichoderma* among the nitrogen sources followed as potassium nitrate ($KNO_3$), ammonium nitrate ($KH_4NO_3$) and ammonium nitrite ($NH_4NO_2$). All species of *Trichoderma* produced dark green and light dark green conidia with prominent concentric rings on $KNO_3$, $KH_4NO_3$ and $NaNO_2$ respectively. *Trichoderma harzianum* HK biomass production was maximum (29.95 mg) among *Trichoderma* strains on nitrogen sources followed by *T. harzianum* HM (22.42 mg), *T. asperellum* TH (19.73 mg) and *T. harzianum* HC (16.55 mg) while all *Trichoderma* strains produced maximum biomass on $NaNO_2$ (41.47 mg) followed by $KNO_3$ (28.72 mg), $KH_4NO_3$ (20.75 mg) and $NH_4NO_2$ (0.00 mg). $NH_4NO_2$ found to be toxic to all *Trichoderma* used strains.

Table 3: Effect of nitrogen sources on biomass production of *Trichoderma* species

| Nitrogen Sources | *T. asperellum TH* | *T. harzianum* HM | *T. harzianum* HK | *T. harzianum* HC | Mean±S.E |
|---|---|---|---|---|---|
| | Mean±S.E | Mean±S.E | Mean±S.E | Mean±S.E | |
| Control | 15.44±0.47 $^{d}$ | 18.22±0.46 $^{d}$ | 22.44±0.67 $^{d}$ | 13.44±0.44 $^{d}$ | 17.39±.51 $^{D}$ |
| $KNO_3$ | 27.56±0.44 $^{b}$ | 29.56±0.56 $^{b}$ | 36.11±0.89 $^{b}$ | 21.67±0.47 $^{b}$ | 28.72±.59 $^{B}$ |
| $NaNO_2$ | 37.44±0.60 $^{a}$ | 44.33±0.93 $^{a}$ | 53.78±0.66 $^{a}$ | 30.33±0.53 $^{a}$ | 41.47±.68 $^{A}$ |
| $NH_4NO_2$ | 0.00±0.00 $^{e}$ | 0.00±0.00 $^{e}$ | 0.00±0.00 $^{e}$ | 0.00±0.00 $^{e}$ | 0.00±0.0 $^{E}$ |
| $KH_4NO_3$ | 18.22±0.46 $^{c}$ | 20.00±0.65 $^{c}$ | 27.44±0.65 $^{c}$ | 27.44±0.41 $^{c}$ | 20.75±.54 $^{C}$ |
| Mean±S.E | 19.733±0.39 $^{C}$ | 22.42±0.52 $^{B}$ | 27.95±0.57 $^{A}$ | 16.55±0.37 $^{D}$ | |

*Small letters are for comparison within column comparison, Capital letters for comparison among nitrogen sources mean, Bold and capital letters are for comparison among species.

The statistical analysis revealed clear differences among *Trichoderma* biomass production on different nitrogen sources in Table 3.

**Effect of pH on biomass production of *Trichoderma* species:** Biomass production of *Trichoderma* spp. was observed at pH 4, pH 5, pH 6, pH7 and pH 8 described in table 4 with three days intervals includes 4 days, 7 days and 10 days. All species produced maximum biomass production at 7 pH followed by 6, 5, 8 and 4 pH value. Biomass production of all species at the end of experiment (at 10 days) was ranged from 926-1128 mg and species showed considerable increase in biomass as time increases at all pH levels. The best pH range was recorded from 6-7 for maximum biomass production. The mass production of *T. asperellum* TH was low as compared to *T. harzianum* rather than *T. harzianum* HC while comparing within *T. harzianum* strains (HM, HK and HC) showed *T. harzianum* HK produced higher biomass followed by *T. harzianum* HM and *T. harzianum* HC. The statistical analysis revealed that there is no significant variation between pH 6 and 7 in table 4.

Table 4: Effect of pH on bio mass production of *Trichodrma* spp.

| pH levels | *T. asperellum* | | | *T. harzianum*HM | | | *T. harzianum*HK | | | *T. harzianum*HC | | | Mass |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4$^{th}$days | 7$^{th}$days | 10$^{th}$days | 4$^{th}$days | 7$^{th}$days | 10$^{th}$days | 4$^{th}$days | 7$^{th}$days | 10$^{th}$days | 4$^{th}$days | 7$^{th}$days | 10$^{th}$days | |
| 4 pH | 411.2±1.8$^{c}$ | 497.0±1.5$^{e}$ | 598.3±1.2$^{e}$ | 425.9±1.2 d | 613.4±1.3$^{c}$ | 704.6±1.9$^{e}$ | 446.5±1.0$^{e}$ | 678.6±1.2$^{c}$ | 787.4±1.3$^{e}$ | 262.2±1.2 e | 431.3±1.0$^{e}$ | 541.4±0.8$^{e}$ | 533.2±1.3$^{B}$ |
| 5 pH | 784.2±2.9$^{c}$ | 886.0±2.4$^{c}$ | 991.0±1.8$^{c}$ | 773.4±1.0 b | 1003.7±1.4 c | 1106.1±1.7 c | 801.7±1.0$^{e}$ | 1033.4±1.5$^{e}$ | 1132.4±1.5$^{c}$ | 701.4±1.5 c | 807±1.3$^{c}$ | 910.0±1.7$^{c}$ | 910.9±1.7$^{C}$ |
| 6 pH | 88.2±3.2$^{b}$ | 1155.3±3.6 e | 1233.4±2.0 b | 985.0±1.1 a | 1199.4±1.7 b | 1290.2±1.2 a | 1013±1.1$^{b}$ | 1272.7±1.3$^{b}$ | 1332.4±1.6$^{a}$ | 782.0±1.3 b | 1039.7±1.7 b | 1134.4±1.3 b | 1110.5±1.8$^{B}$ |
| 7 pH | 989.8±2.3 a | 1268.8±1.9 a | 1325.2±2.2 a | 992.1±1.4 a | 1225.8±1.6 a | 1199.7±1.2 c | 1108.2±1.8 c | 1283.9±1.2$^{a}$ | 1316.7±0.9$^{e}$ | 891.3±1.6 a | 1251.6±1.4 a | 1308.7±1.2 a | 1130.6±1.57 A |
| 8 pH | 490.0±1.4 d | 782.3±3.4$^{d}$ | 847.9±1.4$^{d}$ | 489.9±1.5 c | 817.9±1.46 d | 898.7±1.3$^{d}$ | 503.1±1.1$^{d}$ | 1000.3±1.7$^{e}$ | 1073.0±2.4$^{e}$ | 372.8±1.3 d | 673.1±2.8$^{d}$ | 736.1±1.4$^{d}$ | 723.7±1.8$^{D}$ |
| Mean | 712.6±2.3 | 837.6±2.6 | 998.8±1.7 | 732.8±1.2 | 972.04±1.5 | 109.86±1.4 | 777±1.2 | 1054.78±1.4 | 1128.38±1.5 | 601.9±1.4 | 340.5±1.6 | 926.12±1.4 | |

## DISCUSSION

The physiological parameters play an important role in enhancing mycelial growth and biomass production of *Trichoderma* species its Growth and multiplication of biocontrol agents varies with the substrates (Gao *et al*., 2007; Romero-Arenas *et al*., 2012). Many researchers reported that temperature, pH are key environmental factors that influence Trichoderma bio mass production (Kredics *et al*., 2003; Steyaert *et al*., 2010; Steyaert *et al*., 2010a; Steyaert *et al*., 2010b; Carreras-Villaseñor *et al*., 2012). The influence of pH on biomass and mycelial growth demonstrated that acidic pH is most important key factor for biomass production of all *Trichodemra* species (Singh *et al*, 2014) and they modify the rhizosphere by acidifying the soil which results in unfavorable conditions for growth of plant pathogens (Limón et al., 2004). The present study confirmed that species of *Trichoderma* produced more biomass or grow better in acidic environment. Acidic pH (6.5) is most favorable environment for fungal growth rather than basic or alkaline pH (Limon *et al*., 2004; Singh *et al*., 2014) Our experiments demonstrate that all *Trichoderma* species produce higher biomass production at pH range 5-7 rather than pH 8 and hardly some species of *Trichoderma* seems to be tolerate at pH lower than 3. However, it is more difficult to associate fungal activities in laboratory growth media at low pH with their activities in field conditions or natural habitat where pH effecting microbial activities (Claudia, 2007).

Among environmental parameters, temperature is generally regarded as important factor that also affects *Trichoderma* biomass production. Sharma *et al*., (2005) studied the effect of temperatures and reported that none of the *Trichoderma* species grew at above or at 40 °C. Our study revealed that increase in temperature range from 30 °C reduces the biomass production and 25 °C to 30 °C found to be best for *Trichoderma* growth and biomass production. As temperature seemed to have very adverse effect on the viability of the isolates to produce non-volatile inhibitors and inhibition zone has been found to observe great eat at optimum temperatures which results in increase bio mass production as confirmed by Tronsmo *et al*., 1978; Singh *et al*., 2014.

In additions, selected carbon and nitrogen sources found to be most suitable substrates significantly enhanced growth and population of *Trichoderma*s pecies (Kredics*et al*., 2003). Among the selected carbon and nitrogen sources found to be most effective for growth and multiplication of *Trichoderma* may be due to Fungi use a large number of organic compounds as a carbon sources and about half of the dry weight of the fungus cells consists of carbon, which gives an indication of the important role of carbon compounds within the cell (Singh *et al*., 2012; Rai *et al*., 2016).

**CONCLUSION**

Isolation and morphological characterization are the important tools that leads to further steps for *Trichoderma* biomass production at different nutritional and environmental parameters. A present study was conducted at varying temperature, pH as well as different carbon and nitrogen nutritional sources for biomass production of *Trichoderma* species in order to find out most favorable and relevant parameter. *Trichoderma* biomass and multiplication was most favored by 25°C while 30°C was moderately effective and growth was reduced above 30°C. Correspondingly, pH 7 found to best and biomass production closely related to pH 7 at pH 6 while growth and biomass reduces at pH 8 due to growth of Trichoderma is more efficient in acidic than alkaline soils and they modify the rhizosphere soil by acidifying the soil.

Among the tested sources, mannose, glactose and sucrose found to be most suitable carbon sources for all *T. harzianum* HM, HK, HC and *T. asperelllum* used. Lactose showed no significant biomass as compared to control. Similarly, Sodium nitrate was found to be the best nitrogen source for all *Trichoderma* strains. In the present study, all *Trichoderma* strains produce maximum biomass production on carbon emended media and found to best for its multiplication. *T. harzianum* HK produce maximum biomass on carbon and nitrogen sources at pH and temperature ranges to 5-7 and 25ºC-30ºC respectively. It can be further promoted for production of commercial formulation against soil borne fungal pathogens.

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