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Evaluation of organic culture media for mass production of *Trichoderma harzianum* (Rifai)

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Abstract

Environmental contamination for excessive use of chemical protectant increasing the interest in integrated pest management and organic farming. *Trichoderma harzianum* (Rifai) a promising biocontrol agent fairly acceptable globally used against wide array of plant pathogens. Culture establishment and easy utilization technique of those isolates may not evident for commercialization in all level because of costly production. To cultivate the inoculums in the cheapest suitable media of organic source present study was undertaken. Mycelial growth, conidial production and biomass yield of *Trichoderma harzianum* were examined on four different culture media including potato dextrose agar, modified potato dextrose agar, carrot Agar, pulse sucrose agar. The medium had a significant effect on growth rate and population of the *Trichoderma* species. Carrot Agar was the best medium in terms of quick growth rate and spore production with at low cost. Average linear growth rate was measured after three days of inoculation and highest linear growth has found on potato dextrose agar medium followed by carrot agar. The Biomass yield also recorded as fresh weight and dry weight of inoculums from liquid culture medium and the maximum yielding of inoculums has found in potato dextrose broth and minimum in carrot broth.

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Introduction

Injudicious agrochemicals usage is responsible for wide range of ill effect to agro ecosystem with non-targeted organism. The demand for alternatives to agrochemicals has become stronger owing to concerns about the safety and environmental stress. Researchers are looking for alternative to overcome the adverse effect of chemical reliance. *Trichoderma harzianum* is a well-established bioagent in this regard and readily accessible toolbox for climate smart agriculture. Farmers can easily use these inexpensive inoculums to increase the yield of crop as well as decreasing disease incidence with at low cost.

Trichoderma are free-living filamentous fungi commonly widespread in soil and rhizosp here also found in plant litter, decaying and decorticated wood. Being saprophytic virulent plant symbionts, *Trichoderma* parasitize with other fungi for their opportunistic nature (Harman *et al*., 2004.) It has been acclaimed as an effective, eco-friendly, cheaper, and side effects reducer of synthetic chemicals due to its unique modes of action such as competition, growth promotion, antibiosis and enzymatic. *Trichoderma* induced systemic or local resistance against root and foliar pathogen as well as enhancing plant growth and development by producing plant growth promoting molecules (Shoresh *et al*., 2010) is well defined. There are numerous research conducted on mycoparasitism of *Trichoderma* and found several strain counteract with broad array of commercially important phytopathogenic fungi (Herrera-Estrella and Chet, 2003). This ubiquitous genus is fast growing, inexpensive, easy to cultivation and can produce huge long self-lived conidia which made them usable in certain duration of time with different formulation.

Today *Trichoderma* species are widely used as commercial bioinoculantin biofertilization and biocontroling purpose (Harman, 2006) but not available in local market. Optimization of culture media and quick inoculums production of this antagonist is the first step in utilizing its full potential for specific use.

Culture media were studied for the growth and development of *Trichoderma harzianum* by Elad *et al*., 1981 and Harman *et al.,* 1991, wherein highest conidia produced in Czapek Dox and Richerd's medium but total yields were low. In agar media, potato dextrose agar was the suitable medium producing highest spore and maximum biomass (Mustafa *et al*., 2009). Thus the research paves the way to reach these multi beneficiary bioagentas efficient inoculants to the growers and farmers. Cost effective large scale production, easy and acceptable formulation, establishment in targeted habitat, determining its action in antagonistic arsenal should be another goal needs to future investigation. This study was carried out to explore the effective cheapest organic media from common source and make a comparison of efficacy among four culture media, conidiation with low time regime and to describe their strength and weakness in terms of biomass production.

Materials and methods

Materials

The materials was optimized from collected *Trichoderma harzianum* (Rifai) and cultured primarily on PDA medium. Subcultured the fungus to obtain pure culture of the inoculums. Standard growth stage for media evaluation has found after three days of inoculation. The experiment was setup at the laboratory of Agro technology in the Department of Agronomy and Agricultural Extension of the University of Rajshahi to explore the suitable organic media for quick growth and mass production of *T. harzianum* (Rifai).

Culture establishment and multiplication of the strain

Potato Dextrose Agar medium was made up of the procedure of Anonymous, (1968), a standard principle for *in vitro* fungus culture. Adding and mixingup all ingredients, medium pH then adjusted to 6.0 with 1% HCL in the pH meter. After melting of agar in to the medium was then sterilized in an autoclave at 121°c temperature and 15 psi for 20 minutes. PDA medium was poured in sterilized petridishes at the rate of 20 ml inside the laminar air flow cabinet and allow it to cool down.

A 5mm block of the 3 days old pure culture of *T. harzianum* was cut with flame sterilized cork borer (5mm diameter) and placed on the centre of the medium. After inoculation under aseptic condition in petridishes with inoculums were incubated in a growth chamber at 25±2° temperature. The procedures were further applied for the multiplication of *T. harzianum*.

Culture maintenance and preservation of T. harzianum

Pure culture of *T. harzianum* repeatedly subcultured for proper maintenance of fungal inoculums. Test tube were used as preservation appliance and poured by sterilized PDA medium at 10ml in each tube. The media of test tubes were sterilized in an autoclave at 121°C temperature for 25 minutes. Autoclaved medium in test tube then slanting at 45° angle to increase the surface area. Fungal hyphae of 7 days old culture inoculated in the test tube with the help of a inoculation needle and kept them in the growth chamber at 25±2°C temperature for incubation. The proper growth stage of fungal culture then kept in refrigerator for further use.

Preparation of Different Media

Potato Dextrose Agar (PDA)

The composition and concentration of PDA medium was followed the Anonymous (1968) standard procedure, mention earlier in which 200g potato and 20g dextrose used per 1000ml medium. After mixing, the medium pH was adjusted to 6.00 using 1% HCl with the help of pH meter. As solidifying agent 8g of agar/1000ml were melted in the medium with electric heater. Finally the medium was then sterilized in an autoclave at 121°C temperature and 15 psi for 25 minutes.

Carrot Agar (CA)

400g carrot was weighted, cut into slice and boiled in 1000ml distilled water. Boiled Carrot slice mashed in liquid and sieved into a beaker. The liquid extract made full volume of 1000ml with adding distilled water was then placed on a heater with magnetic stirrer to mix 8g of agar.

The pH of the medium was adjusted to 6.00 using pH meter with the help of 1% HCl. The medium was then sterilized in an autoclave at 121°C temperature and 15psi for 25 minutes.

Modified Potato Dextrose Agar (MPDA)

PDA was further reviewed and modified wherein 150gm potato and 14gm dextrose per 1000ml medium used instead of 200g potato and 20g dextrose respectively. Then pH of the medium was adjusted to 6.00 using 1% HCl with pH meter. Agar melting (8g/1000ml) was occurred by the help of electric heater with magnetic stirrer and the medium was autoclaved as a means of sterilization at 121°C temperature and 15psi for 25 minutes.

Pulse Sucrose Agar (PSA)

40g of pulse was washed well and boiled in distilled water for 1 hr and mesh properly then filter it. After that the pulse extract made up 1 litter with distilled water 10g sucrose add with pulse medium by the help of magnetic stirrer then fixed up the pH of the medium at 6.00 adding with 1% HCl using pH meter.8g Agar was mixed in the medium for solidifying this by electric heater with magnetic stirrer. The medium dispense in conical flask and autoclaved at 121°C temperature and 15psi for 25 minutes.

Pouring of Media

After autoclaving the media, culture vessel and instrument, each petridish were poured with the media at the rate of 20ml, four types of culture media poured similarly inside a laminar air flow chamber. Five petridish for each medium were taken as replicate and marked properly.

Inoculation and Incubation

Fungal culture inoculation has been done when media fully cooled down. Fungal hyphae of three days old pure culture has cut as 5mm disc from the peripheral zone of the colony with the flame sterilised cork borer (5mm). Mycelial block has been placed then at the centre on the medium of Petri dish. 5 mycelial block randomly placed on the 5 replicated plate of each medium.

After sealing the dishes with Para film, all Petri dish were incubated in growth chamber at temperature of 25±2°C and 90% relative humidity (RH) for three days.

Measurement of Average Linear Growth Rate (ALGR) of T. harzianum on Different Growth Media

Linear growth (mm) of *T. harzianum* on the medium of petridish was measured after 1st day of incubation till 3rd day. Three diameters taken from three colonies of each medium then averaging to get Average Linear Growth Rate after 3 days of incubation following the formula (Elad *et al*., 1981).

ALGR (mm/day) = (C3-C0)/3

Where C3= Colony diameter after 3 days of inoculation

C0= Initial colony diameter of inoculation

Colony Characteristics of T. harzianum

Colony characters such as colour of the upper and lower surface of the colony, margin, texture and hyphal density was observed visually in each media after 3 days passing of fungal growth initiation.

Measurement of Fresh and Dry Weight for biomass estimation of T. harzianum on four different media

Liquid culture media or broth media were prepared for the growth of *T. harzianum*in each type of medium.100ml of Potato Dextrose Broth (PDB), Modified Potato Dextrose Broth (MPDB), Carrot Broth (CB), Pulse Sucrose Broth (PSB) were prepared without mixing agar following the formula mention before and dispense in 250ml conical flask.

After cooling the media, 5mm block of 3 days old culture of *T. harzianum* inoculated in all respective types of broth media with replication. The media dispensing and culturing activities were accomplished under laminar air flow cabinet.

The culture flask was incubated in growth chamber at 25±2°C temperature and 90% RH. After 24 hours later of incubation all the conical flask wrecked well with mechanical shaker of 150 rpm speed for 12 hours.

The shaking continued 24 hours interval till 14 days of incubation period to avoid the colony formation and clumping on the surface of the medium. The fungal culture in each conical flask was filtered by Whatman paper No. 1, after 14 days of incubation to separate fungal biomass in each medium. After discarding filtrates each fungal biomass was taken in a filter paper and kept in room temperature for 12 hours and measured fresh weight (mg/100ml) with electric balance.

Then the fungal biomass was dried in hot air oven at 60°C temperature for 24 hours and measured dry weight (mg/100ml) with electric balance for each of the treatment.

Source of materials

T. harzianum (IMI 392432) an isolate, collected from the Laboratory of Microbiology and Biotechnology at the Département of Botany in R.U. which is verified and confirmed by CABI, Bioscience, Surrey, UK.

Experimental Design and Data Analysis

Completely Randomized Design (CRD) with five replications were followed as experimental design in growth measurement from culture media and data were analyzed statistically using MSTAT-C computer program and means were compared following Duncan's Multiple Range Test (DMRT).

Result and discussion

Average Linear Growth Rate (ALGR) of T. harzianum on Four Culture Media

Averase linear growth rate was measured on the basis of Mycelial length spreading to the edge of petridishby *Trichoderma harzianum* (Rifai) to observe the growth phase and maturation with time. Four media was used in this perspective and measured their averase linear rrowth rate in millimeter and calculated the mean value shown in table 1. The average linear growth rate of PDA,

CA, MPDA, PSA media was 27.66 mm, 26.29 mm, 20.77 mm, 17.44 mm/day respectively. ALGR of *T. harzianum* on four tested media was varied significantly (p=0.05).

The highest growth rate was measured 27.66 mm after 1 day in PDA and lowest in PSA that was 17.44 mm. Carrot Agar (CA) medium was in second highest position for mycelial growth of *T. harzianum*. The linear growth rate on MPDA was 20.77 mm/day that was significantly differed with CA and PSA.

Table 1. Mean of average linear growth rate of *T. harzianum*on different culture media.

Culture media	Mean of growth rate with Standard Error
PDA	27.66±.758a
CA	26.29±.758a
MPDA	20.77±.758b
PSA	17.44±.758c

Patterns of linear growth rate of *T. harzianum* on four culture media was placed in Fig. 1 from day 1 to day 3 after inoculation. Mycelium appeared at 1st day after inoculation in carrot agar medium largely in comparison with potato dextrose agar.

In 2nd day after inoculation peripheral growth of mycelium was higher in carrot agar than PDA but 3rd days of inoculation maximum linear growth was observed in PDA. Kumhar *et al.* (2014) optimized different media and explained potato dextrose agar was the best among four lab media.

Spore appeared quickly on carrot agar medium and linear growth limited with the passing of time. N. Subash *et al.* (2013) found growth and sporulation of *Trichoderma harzianum* was best in PDB followed by mineral salts with biogas slurry.

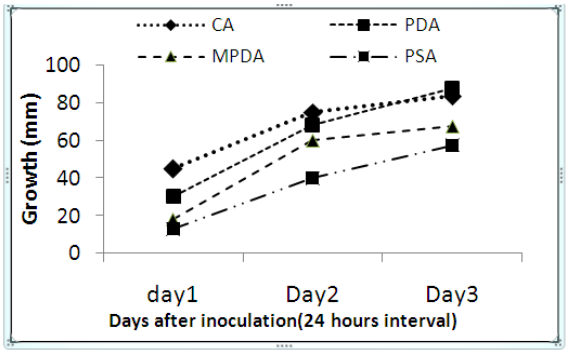

Fig.1. Patterns of linear growth rate of *T. harzianum* from 1st day to 3rd days after inoculation.

Colony Characteristics of T. harzianum on four culture media

Color, margin, texture and hyphal thickness of *T. harzianum* on culture plate were observed as colony characteristics to understand its morphology and aerial growth on 4 different media. The colony colours of *T. harzianum* on agar plate with upper and lower surface were whitish and almost similar in all medium and colony margin was regular in each media. The overall characteristic has shown in table 2. Some differences were observed in terms of texture and hyphal thickness. In PDA media colony texture was compact and hyphae were very thick wherein MPDA media showed moderately compact texture and thin hyphae. Compact colony texture was also found in carrot agar media with moderately thick hyphae whereas loose texture found on PSA media with very thin hyphal arrangement. Jahan *et al.*, 2013. Have found moderately compact texture of *T. harzianum* on PDA plate and loose, puffy texture on MPDA.

Table 2. Colony characteristics of *T. harzianum* on four media.

Culture media	Surface	Color	Margin	Texture	Hyphal thickness
PDA	Upper Lower	Whitish	Regular	Compact	Thick
CA	Upper lower	Whitish	Regular	Compact	Moderately thick
MPDA	Upper Lower	Whitish	Irregular	Moderately compact	Thin
PSA	Upper Lower	Whitish	Irregular	Loose	Very thin

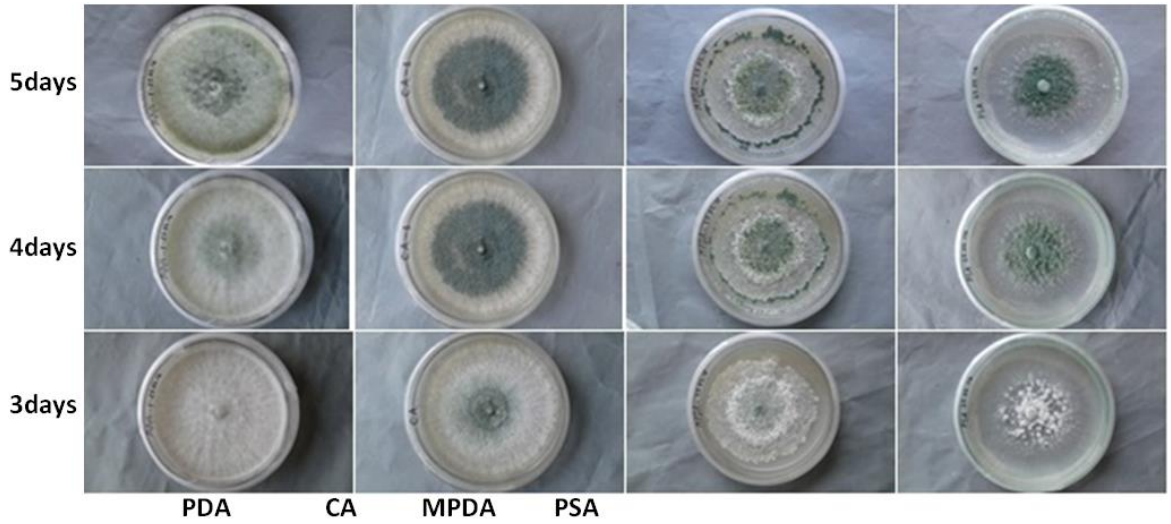

Fig. 2. Growth response of *T. harzianum* on four media after 3 days of inoculation.

Produced Biomass

Developed *T. harzianum* in liquid culture media were weighted freshly and after drying for measuring fresh weight and dry weight to have biomass yield. Among four media fresh weight and dry weight of *T. harzianum* varied significantly (p=0.05). In PDB media the fresh weight and dry weight was 1479.66 and 515.00 respectively whereas 100 ml PSB produced 1320.33mg fresh biomass and 349.66 mg dry biomass. In 100 ml CB media 979mg fresh weight and 131.66 mg dry weight of inoculums has found. The biomass of *T. harzianum* on CB was very poor and varied largely from PDB. The MPDB produced 1220.33 mg fresh biomass and 260.00 mg dry biomass after PSB The highest biomass of *T. harzianum* (Calculated from broth culture) was found in PDB media described at table 3. Sing *et al.*, 2014. reported that maximum biomass produced by *T. harzianum* at 25°C in TSM (*Trichoderma* specific medium) and that was 1420 mg.

Table 3. Biomass weight of *T.harzianum* on different growing media.

Culture media	Fresh weight (mg)	Dry weight (mg)
PDB	1479.66±1.52 a	515.00±0.62 a
PSB	1320.33±1.52 b	349.66±0.62 b
MPDB	1220.33±1.52 c	260.00±0.62 c
CB	979.00±1.52 d	131.66±0.62 d

* In a column, data are the mean values with standard error having different letters within four different culture media differ significantly as per DMRT

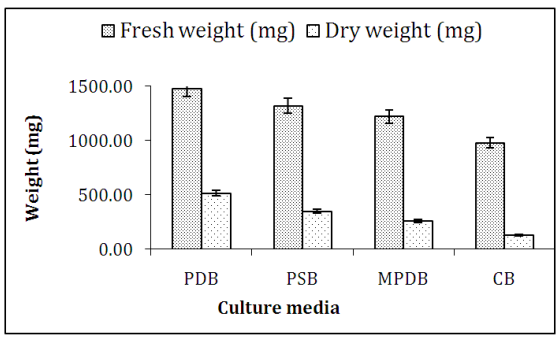

Fig. 3. Comparative presentation of fungal biomass in four liquid media.

Khandelwal and coworkers (2012) found maximum biomass of *Trichoderma* spp. in pulse medium from nine liquid culture media of organic source. The high *Trichoderma* biomass generated in these findings by PDB, PSB produced second highest amount of *T. harzianum* biomass but in agar plate of pulse media produced list biomass unpredictably and the growth was not consistent in all cases. Jahan *et al.* (2013) evaluate five culture media for growth of *T. harzianum* and found 251.20 mg fresh biomass in carrot broth and high biomass measured in PDB.

The studies of Das *et al*., 1997. Showed wheat bran was the best media among four natural media including PDA. The growth was not much higher on carrot agar media in comparison with PDA, but produced second highest mycellial growth and quick maturation (Spore production) occurred on CA medium as early mycellial growth appeared. Liquid culture of carrot media didn't produced expected biomass among four media. It is noted that profuse aerial growth might not resembling the profuse biomass.

Conclusion

It has been found that potato dextrose agar was the best medium for profuse aerial growth of *T. harzianum* wherein MPDA was not satisfactory and carrot agar found better among four medium in terms of cost reduction and quick condition. Biomass production occurred at highest rate in potato dextrose broth, pulse sucrose broth yielded second highest inoculums.

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