Optimization of culture conditions for mass production and bio-formulation of *Trichoderma* using response surface methodology

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Abstract

Use of agro-waste for production of value added products is a good alternative for developing low-cost carriers for formulation of *Trichoderma*-based bio-products. It provides avenues for safe utilization of wastes, while reducing cost and environment pollution load of waste disposal. The present study was undertaken to find suitable agro-waste for economical and higher mass production of *Trichoderma lixii* TvR1 under solid-state fermentation, optimizing culture conditions using mathematical model and assessing effect of formulated bio-product on growth of Spinach (*Spinacia oleracea*). Among various agro-wastes screened, sugarcane bagasse was observed to support maximum growth (20.08×10^7 spores/g) of *T. lixii* TvR1 which was significantly ($p \leq 0.05$) higher than the others. The Response Surface Methodology (RSM) was used to optimize culture conditions using optimal point prediction analysis which predicted that maximum spore production of *T. lixii* TvR1 (19.1245×10^7 spores/g) will be obtained at 30 °C and 68.87% of moisture content after 31 days of incubation. Amendment of formulated bio-product of *T. lixii* TvR1 in soil at concentration 15% w/w promoted biomass, photosynthetic pigments, and protein content of spinach (significant at $p \leq 0.05$). After 6 weeks of sowing the shoot length, root length, and photosynthetic pigments of plants irrigated daily and on alternate days were reported to be increased by 66.97, 185.03, and 82.80%; and 56.56, 71.36, and 74.64%, respectively; over the no amendment.

Keywords Agro-waste · Sub-optimal irrigation · *Trichoderma* · Optimal point prediction analysis · Photosynthetic pigments

Introduction

Trichoderma is outstanding and versatile genus of fungi that has agricultural as well as industrial importance. It possess ability to promote plant growth and productivity, manage pests and pathogens, alleviate abiotic stresses, biodegrade xenobiotic compounds and produce industrially important metabolites (Mastouri et al. 2010; Błaszczyk et al. 2014; Keswani et al. 2014; Hyder et al. 2017). Earlier species of *Trichoderma* were generally considered as biocontrol agents that promote plant growth indirectly by inhibiting growth

of pathogens, however recently many studies supported their direct role in plant growth. Certain beneficial strains of *Trichoderma* release several proteinaceous and auxin-like compounds that modify phytohormones leading to plant growth and development on interaction with plants (Garnica-Vergara et al. 2015; Contreras-Cornejo et al. 2016). The vibrant traits of *Trichoderma*, craft them as a potential candidate for commercial use in agriculture and industries worldwide. For extensive application of *Trichoderma* based products, large scale production of micropropagules is required. The developmental cost and technological challenges are major hindrance for development of successful product (Singh and Nautiyal 2012; Sachdev and Singh 2016). The cost effective large scale production can be achieved through solid state fermentation. Solid state fermentation (SSF) is a cost effective process, widely used for the mass production of filamentous fungi, their enzymes and/or other metabolites on solid substrates with sufficient moisture but not in free-state (Cavalcante et al. 2008). The raw material used as substrate for biomass production accounts 35–40% of production cost (Eltem et al. 2017). Therefore, utilization of

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materials that are cheap, easily available and support extensive growth of beneficial fungi is required for cost effective formulation of product.

The factors that influence the mass production of fungi in SSF include temperature, pH, substrate, and moisture content. The variation in environmental factors affects the growth, sporulation, and germination of the propagules. Therefore, substrates used for mass production of fungi should possess ability to retain large number of germinating fungal propagules, without being affected by minor variation in environmental conditions; simultaneously it should be economically viable, stable, and easily reproducible, whenever required, to make process cost-effective. Hence, the environmental conditions must be optimized for SSF to produce micropropagules on large scale.

The present study was aimed on three steps: (1) screening most suitable agro-waste for mass production of *Trichoderma lixii* TvR1, (2) optimizing culture conditions for maximum mass production of the *T. lixii* TvR1 through SSF using Box–Behnken design of Response Surface Methodology (RSM), which is a mathematical modeling and a statistical tool providing aid for optimizing conditions in a multi-variable system with less number of experiments (Shamshad et al. 2018), and (3) to assess the effect of mass multiplied *T. lixii* TvR1 on spinach growth under optimal and sub-optimal irrigation regimes.

Materials and methods

Fungal strain and substrate collection

Trichoderma lixii TvR1 accession number MF70730 isolated from root of vetiver roots was used in present study (Sachdev and Singh 2018). Five different agro-wastes, viz. vegetable wastes and spent tea leaves collected from hostel mess, Babasaheb Bhimrao Ambedkar (BBA) University, Lucknow, India; sugarcane bagasse collected from local fruit juice vendors; and cow dung and vermicompost collected from local farms were used for mass multiplication of *T. lixii* TvR1.

Mass production of *Trichoderma* on different agro-wastes

The substrates including vegetable wastes and sugarcane bagasse (cut into small pieces < 0.5 cm) were dried under shade. 50 g of each substrate was pre-soaked overnight in sterilized distilled water (SDW) and put into 500 ml conical flask with cotton plug, autoclaved at 121 °C for 15 min. The autoclaved substrates were inoculated with 2 ml of spore suspension (1×10^7 spores/ml) of *T. lixii* TvR1 and incubated at 27 ± 1 °C for 20 days. The experiment was performed in triplicate. The spore density on different substrates was assessed by mixing 1 g of colonized substrate with 9 ml of SDW and agitated at 150 rpm in rotary shaker at room temperature for 1 h. The suspension was filtered through two layers of muslin cloth and spore count was determined by Neubauer's Haemocytometer as spores/g of substrate.

Optimization of temperature, moisture, and incubation time for mass production of *Trichoderma lixii* TvR1 using RSM

A factorial experimental design was carried out to optimize mass production at three levels of temperature (25, 30, and 35 °C), moisture (56.50, 64.97, and 68.87%) and incubation time (5, 10, and 15 days) given in Table 1. 20 g of substrate was taken in conical flask, humidified by adding fixed amount of water. The substrates were autoclaved and inoculated with *T. lixii* TvR1 as mentioned above and incubated at different temperatures in BOD incubator. The spore count was determined at regular interval of time as described above. The growth of *T. lixii* TvR1 was optimized by Box–Behnken model given by Box and Behnken (1960) and computed using quadratic equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j,$$

where Y predicted response, X determining variable, β_0 intercept term, β_i linear effect, β_{ii} squared effect, β_{ij} interaction effect.

Pot experiment

Pot experiment was conducted during April–May, 2015, at environmental field station of BBA University, Lucknow, India, to assess the effect of amendment of *T. lixii* TvR1, mass multiplied on sugarcane bagasse at different concentrations on growth of spinach under optimal and sub-optimal irrigation regimes, i.e., irrigated daily and on alternate days, respectively. *T. lixii* TvR1 mass cultured for one week on sugarcane bagasse was mixed with soil at different concentration viz. 5, 10, and 15% w/w. In control

Table 1 Experimental range of three variables at three levels in Box–Behnken design

Variables	Coded levels		
	– 1	0	1
Temperature A (°C)	25	30	35
Moisture B (%)	56.50	64.97	68.87
Incubation time C (days)	5	10	15

Table 2 Spore density of *Trichoderma lixii* TvR1 on different substrates after 20 days of incubation at $27 \pm 1^\circ\text{C}$

Organic Substrate	Spore density (1×10^7 spores/g)
Spent tea leaves	$3.3 \pm 0.50^{\text{ab}}$
Sugarcane bagasse	$20.05 \pm 1.56^{\text{c}}$
Cow dung	$2.7 \pm 0.35^{\text{a}}$
Vegetable waste	$5.2 \pm 1.58^{\text{b}}$
Vermicompost	$3.2 \pm 0.283^{\text{a}}$

Data are presented as mean of triplicate and values with different letters differ significantly (Tukey's test, $p \leq 0.05$)

setup only soil was used. Further increase in bagasse concentration (beyond 15% w/w) in soil was avoided due to its large volume size. The spinach seeds were procured from local market. The seeds were disinfected with 0.1% HgCl_2 solution for 5 min and then rinsed with SDW for thrice before sowing. The experiments were performed in replicate of three and arranged in complete randomized design.

After 6 weeks of sowing, the plants were uprooted carefully and analyzed for various growth and biochemical parameters including root and shoot length, number of leaves, leaf area, fresh and dry weight of root and shoot, total photosynthetic pigments (Lichtenthaler and Wellburn 1983), and total protein content (Lowry et al. 1951).

Statistical analysis

The experimental data were analyzed statistically using one-way ANOVA followed by Tukey's test at significance level of 5% ($p \leq 0.05$) using Statistical Analysis software SPSS 16.0 Chicago, SPSS Inc. and Microsoft Office 2007. The results presented as mean $\pm$ SD. The mathematical modeling (Response Surface Methodology) was performed using Design Expert software version 9.0.6.

Results and discussion

Mass production of *Trichoderma* on different agro-waste

The result obtained for mass production of *T. lixii* TvR1 on different agro-wastes is presented in Table 2. The data show significantly ($p \leq 0.05$) higher spore production of *T. lixii* TvR1 on sugarcane bagasse as compared to other substrates. The sugarcane bagasse constitutes high cellulosic and sucrose content that supports luxurious fungal growth. Degradation of cellulose helps *Trichoderma* to obtain nutrients effectively (Doni et al. 2014). The sugarcane bagasse also have loose and less compact texture that ease the oxygen

diffusion, nutrients absorption and assimilation by the fungal mycelia (Pang et al. 2006). The strain *T. lixii* TvR1 was found to produce cellulase enzyme (Sachdev and Singh 2018), possessing potential to degrade cellulosic content effectively. The degradation of cellulose fulfills the need of nutrients and presence of sufficient oxygen may be the probable reasons for substantial growth of *T. lixii* TvR1 on sugarcane bagasse. Several workers have reported various other agro-wastes as suitable substrate for mass production of *Trichoderma*. Rama et al. (2001) reported sugarcane bagasse with spent tea leaves as best substrate for growth of *T. reesei* whereas for *T. viride* and *T. koningii*, spent tea leaves + wheat bran and for *T. harzianum*, wheat straw + wheat bran were found as suitable substrates for supporting maximum growth. Analogously, the maximum growth of *T. harzianum* was reported by Mohiddin et al. (2017) on maize seeds supplement with molasses at 10°C . The conidial growth of *T. harzianum* on rice bran was recorded 10.80×10^8 cfu/g, whereas on sugarcane bagasse only 3.73×10^8 cfu/g was documented after 20 days of incubation (Tewari and Bhanu 2004). Similarly, in another study the growth of *T. harzianum* was recorded higher in coir pith (43×10^{10} cfu/g) as compared to sugarcane bagasse (21×10^{10} cfu/g) after 60 days of incubation (Siddhartha et al. 2017). In contrast to the above reports, the study undertaken by Rajput and Shahzad (2015) demonstrated wheat straw and rice husk as less suitable substrate for growth of *T. polysporum*. The dissimilarity in growth of different strains of *Trichoderma* on particular substrate has

Table 3 Box–Behnken design determining factors temperature, moisture level, and time interval for spore density of *Trichoderma lixii* TvR1

Run	A	B	C	Observed value of spores (1×10^7 spores/g)	Predicted value of spores (1×10^7 spores/g)
1	25	56.50	10	3.735	3.835
2	35	56.50	10	2.738	2.434
3	25	68.87	10	7.185	7.492
4	35	68.87	10	5.856	5.759
5	25	64.97	5	0.237	0.063
6	35	64.97	5	0	0.169
7	25	64.97	15	9.158	10.437
8	35	64.97	15	5.452	7.074
9	30	56.50	5	1.18	1.571
10	30	68.87	5	2.5	2.685
11	30	56.50	15	7.138	6.955
12	30	68.87	15	13.212	12.823
13	30	64.97	10	6.033	6.757
14	30	64.97	10	6.033	6.757
15	30	64.97	10	6.033	6.757
16	30	64.97	10	6.033	6.757
17	30	64.97	10	6.033	6.757

Table 4 Analysis of variance (ANOVA) for the experimental results of the quadratic model

Source	Sum of squares	Degree of freedom	Mean square	F value	Prob > F
Model	173.77	9	19.31	126.12	<0.0001 (Significant)
A-temperature	4.91	1	4.91	32.09	0.0008
B-moisture	24.37	1	24.37	159.17	<0.0001
C-time	120.46	1	120.46	786.84	<0.0001
AB	0.028	1	0.028	0.18	0.6841
AC	3.01	1	3.01	19.65	0.0030
BC	5.65	1	5.65	36.91	0.0005
A ²	12.53	1	12.53	81.85	<0.0001
B ²	1.37	1	1.37	8.96	0.0202
C ²	1.50	1	1.50	9.77	0.0167
Residual	1.07	7	0.15	—	—
Lack of fit	1.07	3	0.36	—	—
Pure error	0.000	4	0.000	—	—
Cor total	174.85	16	—	—	—

A, B, C linear model, AC and BC interactive model, A² quadratic model (significance level $p \leq 0.01\%$)
 CV% = 7.51; $R^2 = 0.9939$; Actual $R^2 = 0.9860$; Predicted $R^2 = 0.9019$

been well documented in literature. In our study, cow dung was found to support mass production of *T. lixii* whereas it was reported to be unsuitable for growth of *T. polysporum* (Rajput and Shahzad 2015). These findings suggest that the mass production of *Trichoderma* on different substrates is strain-specific owing ability to utilize carbon and nitrogen as a source of nutrition differently.

Optimization of temperature, moisture and incubation time for mass production of *T. lixii* TvR1

The influence and interaction of all three factors (temperature, moisture, and time) were investigated in 17 runs using Box–Behnken design and the data are presented in Table 3. The predicted values were calculated using coded values of factors through following equation:

$$Y_{\text{spores (spores/g)}} = 6.03 - 0.78A + 1.75B + 3.88C - 0.083AB - 0.87AC + 1.19BC - 1.73A^2 + 0.57B^2 - 0.60C^2.$$

The statistical analysis of obtained data revealed that the model *F* value was 126.12, suggesting that the model is significant. The value of “Prob > *F*” was less than 0.05 indicating model terms are significant (Table 4) and the actual values were more or less similar to the predicted values (Supplementary Fig. 1). The value of CV (7.51%) was found to be low, which determined that the experiment was significant. Overall the result obtained showed that model

was significant and reliable to determine the variable for maximum spore production.

To assess the effect on mass production of *Trichoderma* (behavior of response) by different culture conditions, three-dimensional response surface contour plots were drawn with respect to simultaneous change in two factors at a time (Fig. 1), which determine the interaction effect and optimum level of factors.

The maximum spore production of *T. lixii* TvR1 in all culture conditions studied was found at 30 °C and 68.87% moisture after 15th day incubation (Supplementary Fig. 2) at which spore density was counted 13.21×10^7 spores/g. Cavalcante et al. (2008) have reported 68.41% moisture content favor maximum spore production of *Trichoderma* spp. on wheat bran and further increase in moisture content decreased the conidial count. This result was supported by the study of Rini and Sulochana (2007), who reported maximum spore density of *T. harzianum* and *T. viride* at 60% moisture content on 30th day of incubation on substrates in combination of coir pith and neem cake, and cowdung, neem cake and wheat flour. Moisture content present in SSF affects the physical properties of the substrate. The moisture present cause swelling of the substrates and aid in effective absorption of the nutrients from the substrates that facilitate growth and metabolic activities of fungi (Pang et al. 2006; Cavalcante et al. 2008). Insufficient water content also prevents nutrient accessibility from the substrates by reducing the swelling of substrate which in turn reduce solubilization of nutrients and increase water tension (Pang et al. 2006). Thus, both low as well as high moisture content hinders the growth of fungi in SSF. The optimum temperature that supported

Fig. 1 Three-dimensional response surface plots showing spore density (1×10^7 cfu/g) of *Trichoderma lixii* TvR1 (R1) under the effect of; A—moisture and temperature; B—time and temperature; C—time and moisture

maximum growth of *T. lixii* TvR1 was 30 °C. This result was comparable with the results reported by Shahid et al. (2011) where 25–30 °C was observed as optimum temperature range for fast growth of *Trichoderma* spp. However, the study undertaken by Mohiddin et al. (2017) 10 °C was found as most favorable temperature, supporting maximum growth of *T. harzianum* compared to 20 and 30 °C. In our study, after 15 days of incubation, *T. lixii* TvR1 was still found in growth phase which indicated further increase in incubation time could result in more spore production. The maximum spore production (19.1245×10^7 spores/g) was predicted to be obtained after 31 days of incubation using optimal point prediction analysis under similar condition of temperature and moisture. This finding corroborates with results of Rini and Sulochana (2007), who reported maximum spore density on 30th day after incubation.

Growth stimulating role of mass multiplied *T. lixii* TvR1 on spinach

The data presented in Fig. 2a–j show positive relation between spinach growth and increase in concentrations of *T. lixii* TvR1. The maximum growth and biochemical

parameters were reported for spinach plants grown in soil amended with 15% w/w *T. lixii* TvR1 formulated biofertilizer under both irrigation regimes and were found significantly higher ($p \leq 0.05$) than their respective controls. However, plants subjected to alternate day irrigation showed slightly lower values for growth parameters as compared to plants irrigated daily. Increase in sugarcane bagasse concentration in soil could positively have stimulated the plant growth; however owing to large volume its higher concentration was not taken in present study. This problem can be resolved by increasing concentration of *Trichoderma* inoculums in sugarcane bagasse. The increased growth of spinach in all treatments as compared to control highlights the positive effect of *Trichoderma* in plant growth promotion representing *Trichoderma* as Plant growth promoting fungi (PGPF) effective even under sub-optimal water condition. The increase in growth parameters of maize plant with increase in concentration of *Trichoderma* has been reported by Akladios and Abbas (2014). This increase may be contributed by the ability of *Trichoderma* spp. to increase the solubility of insoluble plant nutrients and enhanced uptake by plants and by production of various organic acids that reduce soil pH (Kaya et al. 2009; Akladios and Abbas 2014). Yedidia

Fig. 2 Growth and Biochemical parameters of Spinach plants grown in soil amended with different concentration of *Trichoderma lixii* TvR1 mass multiplied on bagasse under different irrigation regimes after 6 weeks of sowing. **a** Shoot length; **b** root length; **c** number of leaves; **d** leaf area; **e** fresh shoot weight; **f** dry shoot weight; **g** fresh

root weight; **h** dry root weight; **i** total photosynthetic pigment in mg/g Fwt; and **j** protein content in mg/g Fwt. Data are presented as mean and values with different letters differ significantly (Tukey's test, $p < 0.05$) ($n = 3$)

et al. (2001) reported significant increase in shoot length, plant dry weight and leaf area of cucumber plants colonized by *T. asperellum* due to enhanced availability of Phosphorus and Iron. Availability of nutrients to plants grown in *Trichoderma* amended soil may be the reason for increase in plant height, leaf number and area and fresh and dry weight in present work. The *Trichoderma* spp. are also known to produce phytohormones like auxin and gibberellins that promote lateral root formation and water uptake efficiency from soil (Contreras-Cornejo et al. 2009; Martinez-Medina

et al. 2011; Doni et al. 2014). *Trichoderma* spp. also possess ability to buffer environmental conditions such as drought, pH, flooding, etc. through action of various mechanisms (Doni et al. 2014). Mastouri et al. (2010) and Shukla et al. (2012) observed increased uniformity in seed germination and seedling growth of tomato; and water holding capacity of rice plants, respectively under water deficient condition on inoculation with *T. harzianum*. Similarly, *T. hamatum* isolate DIS 219b was found to delay drought response in coco seedling, probably due to the enhanced root growth

that improved water status (Bae et al. 2009). These findings are analogous to the findings of present study, demonstrating stimulation of root size of plant may be due to production of phytohormones that strengthens the capacity of plant to anchor more firmly deep into soil and obtain maximum water and nutrients, enhancing physiological processes of plant and resulting in good plant growth and development even under sub-optimal water condition.

Total photosynthetic pigments and protein content were also observed to be increased significantly ($p \leq 0.05$) with increase in *T. lixii* TvR1 and were found comparatively higher in plants irrigated regularly than corresponding alternate day irrigated plants (Fig. 2i). Contreras-Cornejo et al. (2009) and Akladios and Abbas (2014) in their study observed the significant increase in photosynthetic pigment in plants treated with *Trichoderma*. Shores (2010) and Vitti et al. (2016) reported enhanced greenness and photosynthetic activity in maize and tomato plants, respectively, on inoculation with *T. harzianum*-T22 which may be attributed by reprogramming of plant gene expression. Prolonged photosynthetic activity in rice plants was found to be related with the increased root growth and distribution, attributed by the activity of *Trichoderma* spp. (Mishra and Salokhe 2011). Increase in protein content in plants may be due to increased uptake of nitrates and other ions. *Trichoderma* on cellulose degradation release large amount of nitrogen (N) in soil that contributes to high photosynthetic rate (Doni et al. 2014) and protein content (Subramanian et al. 2006) due to increased N uptake by plant. Even *Trichoderma* spp. are known to enhance nitrogen use efficiency in plants that results in greater yields (Shores 2010). These observations suggest degradation of sugarcane bagasse by *T. lixii* TvR1 may result in release of N in soil that positively stimulated photosynthetic and protein content by increasing availability and uptake of N by the spinach plants.

Conclusion

The study concluded that sugarcane bagasse was an efficient agro-waste for mass production of *T. lixii* TvR1. The sugarcane bagasse was a good source of nutrition that resulted in the significant mass production of *T. lixii* TvR1 in absence of any additional supplement and retained viable spores. The study also highlighted the fact that the growth of *Trichoderma* on different agro-wastes could be strain specific. The application of mathematical modeling was a significant approach to optimize the culture conditions with less number of experiments. The results of mathematical modeling indicate its applicability to predict the growth rate of *T. lixii* TvR1 under different culture conditions. Therefore, this approach could be used to predict impact of controlled

environmental conditions on growth rate of fungus and assist in formulation of commercial product. The formulated bio-product effectively promoted the growth of spinach under normal and sub-optimal irrigation condition revealing that the utilization of agro-waste as carrier for formulation of *Trichoderma*-based bio-product is an economical and safer alternative under prevailing sub-optimal environmental conditions.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest.

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