



Exploration of indigenous *Trichoderma* species for their use as biofertilizers, optimization of growth conditions and cultivation on cheap substrates

Dhurva Prasad Gauchan^{1*}; Ashok Kumar Bhattarai²; Shishir Pandey¹; Sunil Bhandari¹

¹Department of Biotechnology, School of Science, Kathmandu University, Dhulikhel-45200, Kavrepalanchok District, Bagmati, Nepal; ²Praramva Biotech Pvt. Ltd., Nagarjun-6, Kathmandu, Nepal

*Corresponding author E-mail: gauchan@ku.edu.np



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Abstract

Members of the genus *Trichoderma* have substantially improved the agricultural economy through inhibiting the phytopathogens, assimilating nutrients and inducing defensive metabolism against the environmental stresses. The major aim of this study was to isolate indigenous *Trichoderma* that can be used as a potential biocontrol agent (BCA). Currently, we isolated 14 native fungal isolates from vermicompost, agricultural soils and infected substrates, and identified them as *Trichoderma* spp. based on their morphological characteristics. Isolates designated V1D, V1F, V3D, V3F, W1, W2, KAL, NAR, BIOC, AG, RD and NIM resembled to *T. viride*, while MUSH and RF resembled *T. harzianum*. The ability of these isolates to assimilate different forms of sugars, nitrogen source (N) and phosphates (PO₄) were evaluated qualitatively. Isolates showed differing solubilization zones (cm) in agar plates containing cellulose (1.8-9.5 cm), amylose (0.1-2.1 cm), tri-calcium phosphate (0.1-0.17 cm) and di-calcium phosphate (0.33-0.53 cm), to substantiate their biofertilizer potentialities. Confrontation assay with dual culture technique against seven phytopathogens (i.e. *Rhizoctonia solani*, *Aspergillus niger*, *Sclerotinia sclerotiorum*, *S. rolfsii*, *Fusarium solani*, *F. oxysporum* and *Botryodiplodia theobromae*) revealed promising mycoparasitic activity. Three isolates (MUSH, BIOC and V3F) showed mycelial growth inhibition in the range of 33-77%, compared to the control plate (without isolates). With respect to isolate MUSH, a significantly higher (P< 0.05) dry biomass weight (g) was obtained at pH 7 (0.66 ± 0.05) and pH 6 (0.55 ± 0.05), than at pH 3, pH 4 and pH 5. Similarly, higher biomass significance (P< 0.001) was obtained in yeast mannitol broth (2.58 ± 0.11 g), compared to potato dextrose broth (PDB) and nutrient broth (NB). The production of spores by isolate MUSH was tested on four locally available solid substrates (i.e. corn stalk, rice husk, jackfruit molasses and sugarcane bagasse) through solid state fermentation (SSF). Production of conidia (cfu/ g) was higher in corn stalk (72.6 × 10⁸), followed by rice husk (68.4 × 10⁸), jackfruit molasses (18.6 × 10⁸) and sugarcane bagasse (12.4 × 10⁸). High counts of conidia production on these substrates render MUSH isolate efficient to be used as a BCA by the farmers, to enhance their crop productivity.

Keywords: *Trichoderma* spp., Biocontrol agent, Biofertilizer, Confrontation assay, Solid state fermentation

1. Introduction

Annually, huge loss of the agricultural economy has been reported throughout the world ([Savary *et al.*, \(2012\)](#)). Several biotic and abiotic factors were found to be responsible, among which considerable loss of agricultural crops incurred by the pathogenic infestation is of growing concern. [Spiegel and Chet, \(1998\)](#) highlighted that biological control of plant pathogens with *Trichoderma* spp. is a part of integrated disease management technique. [Druzhinina *et al.*, \(2011\)](#) reported that *Trichoderma* is a ubiquitous genus, free-living, filamentous, ascomycetous fungus widely used as a BCA against several economically important soil-borne, fruit, foliar plant pathogens and nematodes. Later, [Harman *et al.*, \(2004\)](#); [Saravanakumar *et al.*, \(2016\)](#) added that members of *Trichoderma* exhibit induced localized and systemic resistance in wide variety of crops, legumes and vegetables against several pathogens including; *Xanthomonas oryzae*, *F. graminearum*, *Botrytis cinerea*, *R. solani*, *Alternaria solani*, *Phytophthora capsici*, Green-mottle mosaic virus etc.

Recent studies of [Mukherjee *et al.*, \(2013\)](#); [Contreras-Cornejo *et al.*, \(2016\)](#) revealed that mycoparasitism, antibiosis and rhizosphere competence have been long established as antagonistic mechanisms, regulated through specialized molecular orchestration in *Trichoderma* spp. [Harman *et al.*, \(2004\)](#) highlighted that synthesis of peptides, proteins and low-molecular weight compounds are considered as the three major arsenals primarily involved in eliciting plant defense responses. An antifungal agent called harzianic acid isolated from *T. harzianum* strain was shown to have growth regulating effect, in addition to its parasitic mode of action ([Vinale *et al.*, \(2013\)](#)). Moreover, [Vinale *et al.*, \(2008\)](#) added that *Trichoderma* spp. are effective root colonizers, interact with the plants and provide tolerance to biotic and abiotic stresses to ultimately enhance crop productivity. Generally,

Trichoderma spp. colonize roots, however the plant protection benefits could also be obtained by applying them on fruits, flowers and foliage ([Harman, 2000](#)). In addition, *Trichoderma* spp. induced plant resistance against abiotic stresses such as; drought, salinity, osmolarity, heat and cold, as reported by [Shoresh *et al.*, \(2010\)](#).

According to [Harman *et al.*, \(2004\)](#), *Trichoderma* strains are prolific producers of enzymes which subsequently have its implications on the uptake of Phosphorus (P), Nitrogen (N) and in the chelation of minerals such as Fe³⁺, Mn⁴⁺, Cu²⁺, Zn etc. Recently, [Li *et al.*, \(2015\)](#) studied the N and P assimilation abilities of the *Trichoderma* spp., and promising results were obtained for their potential use as biofertilizers. In a recent study conducted by [Poveda *et al.*, \(2019\)](#), co-inoculation of *T. harzianum* with arbuscular mycorrhizal fungi (AMF) was shown to favor the presence of AMF in the roots of non-host Arabidopsis and rapeseed plants.

The innovative technology in the areas of agriculture improved crop production, but some modern practices have debilitating impacts on the environment. This demands us to adopt and develop eco-friendly farming solutions for sustainable agriculture. Optimized production of the BCA's by modulating pH, water, media, temperature and light, is imperative for their commercial production. [Cavalcante *et al.*, \(2008\)](#) reported that cultivation of the *Trichoderma* bioagents in a cheaply available solid medium through SSF provides a robust and low-cost platform for the production of fungal biomass. Later, [Bashan *et al.*, \(2014\)](#) added that many biopesticides with *Trichoderma* supplements are produced commercially and the use of these kinds of products are gradually increasing in developing countries. However, not all species of *Trichoderma* are equally antagonistic, thus there is a strong need to explore the most potent BCA. An early study of [Ommati and Zaker, \(2012\)](#) revealed that indigenous *Trichoderma* spp. are more effective

biogents, as they are adapted to their natural environment. Considering these facts, the objectives of the present study were to investigate and evaluate the use of endemic *Trichoderma* spp. as BCA's, in addition to their possible use as biofertilizers for soil and vermicompost amendments.

2. Materials and Methods

2.1. Sample collection

Different types of samples including; compost, vermicompost, soil, mushroom bulb, peel of lemon and jackfruit were collected from major agricultural pockets of the country such as; Kathmandu, Bhaktapur, Chitwan, Rupandehi and Kavre. The samples were taken from a depth of 15 cm of the agricultural land, collected in sterile polyethylene bags, transported to the laboratory and then stored at 4°C until use. Details of sample sources and their respective locations are provided in Table (1).

2.2. Isolation of *Trichoderma* spp.

Samples showing mold-like infection in the mushroom bulb, jackfruits and lemon peels were collected from the fields, and visualized under bright field microscope to observe fungal growth. The infected parts were rinsed under tap water and then placed in 0.1% HgCl₂ for 10 min. for surface sterilization, followed by rinsing for 3 to 4 times with sterilized dist. water to remove the residual HgCl₂. The infected parts were placed aseptically on the surface of potato dextrose agar (PDA) medium, and incubated at 28± 2°C for 7 d ([Rahman *et al.*, 2011](#)). Isolation of *Trichoderma* from compost, vermicompost, and soil was carried out using the multiple-tube dilution technique on malt extract agar supplemented with chloramphenicol (0.25 g/ l) and rose bengal (0.15g/ l) according to [Rahman *et al.*, \(2011\)](#). Plates were incubated at 28°C for 7 d. The emerging conidia from fungal bodies were isolated, and then stored on potato dextrose agar (PDA) slants at 4°C.

2.3. Morphological characteristics

All isolates were grown on PDA for morphological analysis. Comparison of the morphological structure and classification of all the fungal isolates into *T. harzianum* and *T. viride* was carried out using the identification key provided by [Shah *et al.*, \(2012\)](#). Isolates were observed under a bright field microscope and distinguished based on the colony shape and color, conidiophores arrangement, conidia shape and color, and shape of phialides.

2.4. Biochemical characterization of the isolates

2.4.1. Carbohydrate assimilation test

The test was carried out once using basal media composed of; 8.4 mM NH₄H₂PO₄, 2.7 mM KCl, 0.8 mM MgSO₄.7H₂O, 0.035 mM ZnSO₄.7H₂O and 0.02 mM CuSO₄.5H₂O, in reference to the slightly modified method of [Kitancharoen and Hatai, \(1998\)](#). Briefly, 50 mg/ l of bromocresol purple dye was added to the basal medium, and then filter-sterilized (0.45 µm, Thermo Fisher Scientific, USA), simple sugars (i.e. glucose, fructose, mannitol, sucrose, cellobiose, galactose, xylose and arabinose), and complex sugars (i.e. pectin, xylan, cellulose, carboxy-methyl cellulose (CMC) and starch) procured from HIMEDIA® (India) were added separately to each tube at a final concentration of 1% (w/v), pH was maintained to 7.0. About 2 ml portions of the medium were poured into 10 ml sterilized test tubes. Tubes were inoculated individually with mycelia of all the fourteen fungal isolates, and then incubated at 28°C for 7 d. Tubes containing basal medium supplemented with each sugar individually were taken as controls. After incubation, change of the color from purple to yellow or orange was considered as a positive result. Three replicates were used for each sugar source, and the assay was repeated twice.

2.4.2. Detection of the cellulolytic and amylolytic activity of the isolates

Qualitative analysis of cellulase activity of the *Trichoderma* isolates was carried out three times in a

single replication, in reference to [Kitancharoen and Hatai, \(1998\)](#). The same basal medium used for carbohydrate assimilation test was used with the addition of 1% (w/v) micro-granular cellulose (Sigma-Aldrich, USA) instead of the sugar, and 1.2% (w/v) agar. On the other hand, the amylolytic potential was tested thrice in a single replication using PDA as the basal media with the addition of 2% (w/v) starch. The media were autoclaved and then 20 ml of each medium was poured into the petri dishes. Each of the tested

Trichoderma isolates was inoculated individually in the form of a longitudinal streak onto the center of the agar plates, and then the cultures were incubated for 7 d at 28°C. The presence of halo zones around the fungal streaks for cellulase and amylase activity was detected after incubation. Visualization of clear zone for amylase activity was done by flooding the plates with Iodine solution composed of: 1% (w/v) KI and 0.3 % (w/v) I₂, according to [Marlida *et al.*, \(2000\)](#).

Table 1. Details of samples collection sites, sources and designated isolates symbols

Isolates	Sources	Locations
V1D	Horse manure vermicompost	Kathmandu, PBPL
V1F	Horse manure vermicompost	Kathmandu, PBPL
V3D	Cow dung vermicompost	Kathmandu, PBPL
V3F	Cow dung vermicompost	Kathmandu, PBPL
W1	Wine substrate from jackfruit	Kathmandu University, DoBT
W2	Infected peel of jackfruit	Kathmandu University, DoBT
KAL	Soil from tomato farm	Chobhar, Kathmandu
NAR	Vermicompost	Narayangad, Chitwan
BIOC	Soil from pea farm, formerly treated with <i>Trichoderma</i> from BIPL	Budol, Kavre
AG	Soil from cauliflower farm, formerly treated with <i>Trichoderma</i> from ANPL	Ramkot, Kathmandu
MUSH	Mushroom substrate from FAPL	Nalinchok, Bhaktapur
RF	Compost of LAOFC	Tikuligarh, Rupandehi
RD	Compost of LAOFC	Tikuligarh, Rupandehi
NIM	Rotten lemon (nimbu)	Kalanki, Kathmandu

Abbreviations: PBPL (Prarambha Biotech Pvt. Ltd.), BIPL (Biocure India Pvt. Ltd.), ANPL (Agricare Nepal Pvt. Ltd.), FAPL (Farmland Agro Pvt. Ltd.), LAOFC (Lumbini Agro Organic Fertilizer Company), DoBT (Department of Biotechnology)

2.4.3. Phosphate utilization assay

According to the method adopted by [Zaidi *et al.*, \(2009\)](#), qualitative estimation of phosphate solubilization by *Trichoderma* isolates was done based on their ability to form clear zone around the isolates in the Pikovskaya's medium ([Pikovskaya, 1948](#)) consisting of (g/ l): yeast extract, 0.5; dextrose, 10; (NH₄)₃PO₄, 0.5; KCl, 0.2; MgSO₄, 0.1; MnSO₄, 0.0001; FeSO₄, 0.0001, agar, 15 and tri-Calcium Phosphate (TCP), 5 as a sole source of P. Solubilization of the dibasic-Calcium Phosphate (DCP) was tested in the same medium constituents with replacing TCP by 5 g/ l DCP. The isolates were incubated at 28°C for 7 d for observation of the halo zones ([Zaidi *et al.*, 2009](#)). We conducted three independent experiments in a single replication.

2.4.4. Nitrogen assimilation and urease activity assay

Briefly, 10 ml of a mineral solution containing 0.6 M KCl, 0.2 M MgSO₄ and 3.6 mM FeSO₄ was added to the mixture of 5.7 mM K₂HPO₄, 0.30 % (w/v) sucrose and 1.2 % (w/v) agar. The resulting mixture was supplemented with 5 mM NH₄NO₂ or 5 mM KNO₃ to detect NO₂ assimilation ([Kitancharoen and Hatai, 1998](#)). Utilization of NaNO₃ was tested by cultivating the fungal mycelia in Czapek's Dox Agar (CZDA) medium. The media were autoclaved and then a 10 ml portion was poured individually into the petri plates. The fungal isolates were inoculated in the form of a longitudinal streak at the center of the plates, and incubated at 27°C for 7 d to observe their ability to grow. For detecting the urease activity, Stuart's urea broth was used consisting of; yeast extract (0.1 g/ l), K₂HPO₄ (9.5 g/ l), KH₂PO₄ (9.1 g/ l) and phenol red (0.01 g/ l) ([Stuart *et al.*, 1944](#)). The medium was autoclaved and filter-sterilized urea (0.45 µm, Thermo Fisher Scientific, USA) was added to a final concentration of 20 g/l. Fungal mycelia were inoculated individually and then incubated at 27°C for 7 d.

After incubation, urease production is indicated by the development of a bright pink color throughout the broth. The assays were conducted only once.

2.5. *In vitro* antagonism assay

Analysis of the *in vitro* antifungal potential of *Trichoderma* isolates against seven plant pathogenic fungi, namely, *R. solani*, *A. niger*, *S. sclerotiorum*, *S. rolfsii*, *F. solani*, *F. oxysporum* and *B. theobromae* were carried out using dual culture assay ([Kotasthane *et al.*, 2015](#)). These pathogenic cultures were kindly provided by Nepal Academy of Research Council (NARC), Department of Plant Pathology, Kathmandu, Nepal, from their culture collection. A 5 mm diameter disc of each *Trichoderma* isolate was inoculated on the surface of PDA plate, 1.5 cm away from the margin of the petri plate. The fungal pathogens were placed individually opposite to the *Trichoderma* disc, at 1.5 cm away from the margin of the petri plate. In control plates, a sterile agar disc was placed at the opposite side of the fungal pathogen. The plates were incubated at 28°C for 7 d. The assay was repeated thrice in a single replication. The percentage (%) of radial growth inhibition of each pathogen was measured using the following formula:

$$\% \text{ Inhibition of radial mycelial growth} = [(C-T)/C] \times 100$$

where, C is the radial growth measurement of the pathogen in the control plates, and T is the radial growth of the pathogen in presence of *Trichoderma* isolates, according to [Kotasthane *et al.*, \(2015\)](#).

2.6. Optimization of growth conditions for the MUSH isolate

2.6.1. Growth in different pH values

Growth of the MUSH isolate on media having five different pH values (i.e. 3, 4, 5, 6 and 7) was analyzed, to determine the optimum pH giving maximum growth. Briefly, a 5 mm mycelial disc of

the MUSH isolate was aseptically and individually inoculated into a 50 ml potato dextrose broth (PDB), maintained at different pH levels by the addition of 1 M tartaric acid and 1 M NaOH. These seeded media were incubated at 27°C for 10 d ([Reetha *et al.*, 2014](#)). The biomass yields of each *Trichoderma* isolate were filtered in Whatman no. 1 filter paper, and then dried in an oven at 60°C. The dry yields of the fungal biomass were weighted using a digital balance (CAH-123, CONTECH®, India). For each pH treatment, three independent assays in a single replication were performed.

2.6.2. Growth in different synthetic broth media

The growth of the MUSH isolate was studied using three broth media viz., nutrient broth (NB), PDB and Yeast mannitol broth (YMB) at the optimum predetermined pH level. NB was used in this assay because it does not contain simple sugars, which are generally the preferred sugar sources for *Trichoderma* spp. ([Manczinger and Pollner, 1987](#)). These three broth media were purchased from Himedia Pvt. Ltd., India. Preparation of fungal starter culture was also carried out in those respective media. A 5 mm mycelial disc was aseptically inoculated in 50 ml of each medium, and then incubated at 28°C for 15 d, with a slow agitation at 150 rpm. The dry yields of the fungal biomass were separated from broth by filtration using Whatman no. 1 filter paper, dried at 60°C, and finally weighted using a digital balance (CAH-123, CONTECH®, India). For each broth medium, the assay was conducted thrice with a single replication.

2.6.3. Growth in different solid media

Solid state fermentation (SSF) of MUSH isolate was performed only once using four different inexpensive and locally available solid substrates including; rice barn, sugarcane bagasse, cornstalk and molasses of jackfruit. Following the modified method of [Cavalcante *et al.*, \(2008\)](#), these substrates were grinded to small particles using mortar and pestle. About 40 g of each substrate was dissolved in

40 ml of dist. water and then incubated overnight for humidification. After incubation, the pH of the substrates was measured and excess water was drained out. These substrates were autoclaved at 121°C for 20 min. without any nutrient supplementation. The MUSH isolate was grown on PDA slant. After 7 d of incubation at 28°C, the green conidia were harvested by adding suitable volume of 0.01% (v/v) sterile Tween-80 (HIMEDIA®, India). Briefly, 40 g of each solid substrate was inoculated individually with 1 ml of Tween-80 solution containing $\sim 1 \times 10^6$ conidia inside 250 ml Erlenmeyer's flasks, followed by incubation for 14 d at 28°C. After incubation, the conidia were harvested by adding three portions of 50 ml of 0.01% (v/v) sterile Tween-80. Approximately 10 μ l of the suspension was pipetted out, and then the green conidia were counted using a hemocytometer under bright field microscope. Results were expressed as colony forming units (cfu) per g of dry solid (gds).

2.7. Statistical analysis

Data were represented as mean \pm standard deviation (SD). Multiple pairwise comparisons were carried out with one-way analysis of Variances (ANOVA), followed by contrast procedure with Turkey Honestly Significant Difference (HSD) test, when appropriate at alpha level of 0.05. Outliers in the data from normal distribution were evaluated with quantile-quantile plot. *p*-value less than 0.05 was considered statistically significant. Asterisk (*) sign was assigned to show the significant difference in the mean values. Statistical analyses and graphs were constructed using RStudio ver. 1.2.5033 ([Allaire, 2016](#)).

3. Results

3.1. Morphological identification of the fungal isolates

About fourteen distinct *Trichoderma* isolates designated as; V1D, V1F, V3D, V3F, W1, W2, KAL, NAR, BIOC, AG, MUSH, RF, RD and NIM

are recovered from vermicompost, agricultural soils and infected substrates such as; mushroom, jackfruit and lemon. These isolates are identified according to their phenotypic characteristics. The color and shape of the mycelia, shape of phialide, conidiophores arrangement, conidia shape and color are observed on PDA after 7 d of incubation. Most of the isolates namely V1D, W1, W2, KAL, NAR, BIOC, AG, MUSH, RD appeared dark green in color. V3D and V3F appeared bluish green, while V1F and RF appeared yellowish green in color. Only one isolate designated as NIM appeared glossy green in appearance. Three isolates viz., MUSH, V1F and RF showed concentric growth, whereas the other

isolates appeared irregular and circular on PDA (Table 2). Dense and light green conidia are produced by all the isolates. Conidiophores are either loosely or compactly arranged. Two isolates viz., MUSH and RF have globose to sub-globose conidia and flask-shaped phialides. The remaining twelve isolates have globose to ellipsoidal conidia and slender shaped phialides. Based on these phenotypic characteristics twelve isolates (viz. V1D, V1F, V3D, V3F, W1, W2, KAL, NAR, BIOC, AG, RD and NIM) resembled to *T. viride*, while two isolates (viz. MUSH and RF) resembled to *T. harzianum*.

Table 2. Morphological evaluation of 14 *Trichoderma* isolates based on their colony color, colony appearance, conidiophore arrangement, phialide shape and conidia color and shape.

Isolates	Colony color	Colony appearance	Conidiophore arrangement	Conidia shape	Conidia color	Phialide shape	Resemblance
V1D	dark green	irregular	compactly arranged	globose	light green	slender	<i>T. viride</i>
V1F	yellowish green	concentric circle	loosely arranged	globose	light green	slender	<i>T. viride</i>
V3D	bluish green	circular	compactly arranged	globose to ellipsoidal	light green	slender	<i>T. viride</i>
V3F	bluish green	circular	loosely arranged	globose	light green	slender	<i>T. viride</i>
W1	dark green	irregular	compactly arranged	globose	light green	slender	<i>T. viride</i>
W2	dark green	irregular	compactly arranged	globose to ellipsoidal	light green	slender	<i>T. viride</i>
KAL	dark green	irregular	compactly arranged	globose	light green	slender	<i>T. viride</i>
NAR	dark green	circular	loosely arranged	globose	light green	slender	<i>T. viride</i>
BIOC	dark green	circular	compactly arranged	globose to ellipsoidal	light green	slender	<i>T. viride</i>
AG	dark green	irregular	compactly arranged	globose	light green	slender	<i>T. viride</i>
MUSH	dark green	concentric circle	compactly arranged	globose to sub-globose	light green	flask-shaped	<i>T. harzianum</i>
RF	brownish/ yellowish green	concentric circle	loosely arranged	globose to sub-globose	light green	flask-shaped	<i>T. harzianum</i>

RD	dark green	circular	loosely arranged	globose	light green	slender	<i>T. viride</i>
NIM	glossy, green with white filament	circular	compactly arranged	globose	light green	slender	<i>T. viride</i>

3.2. Carbohydrate assimilation assay

All the fourteen isolates assimilated the simple sugars including; glucose, fructose, mannitol, sucrose, cellobiose and galactose. With the exception of RD, all isolates are able to utilize xylose. Five isolates viz., V1D, V3F, BIOC, AG and RF are not able to utilize arabinose. In case of the complex sugars, cellulose and amylose are utilized by all the isolates giving evidence of cellulolytic and amylolytic activities, respectively. However, Carboxy-methyl cellulose (CMC) is not hydrolyzed by five isolates namely; V1D, V3F, NAR, BIOC and MUSH. Pectin is hydrolyzed by only four isolates viz., V1D, V1F, V3D and NIM. None of the fourteen isolates are able to degrade xylan. Four isolates viz., NIM, V3D, V1F and V1D are able to hydrolyze all the complex carbohydrates except xylan.

3.3. Cellulase and amylase activities

For the qualitative estimation of the cellulase and amylase activities, a clear halo zone diameter is measured carefully around the fungal streak. All the 14 isolates showed some degree of cellulose solubilization activities giving halo zone diameters ranging from 1.8 to 9.5 cm, compared with the control. Higher amount of cellulase is recorded by the isolates viz., V3F, RF, MUSH, BIOC and NAR, where clear halo zones diameters of 8.5 to 9.5 cm are observed for these isolates, indicating degradation of almost all the cellulose in the petri plates. Cellulose solubilization zone for the isolates is presented in Fig. (1a). Starch assimilating abilities of all the *Trichoderma* isolates is measured by

measuring diameters of the clear zone produced after treating the petri plates with Iodine solution. All the isolates are found to have some degree of solubilization producing degradation diameters ranging from 0.1 to 2.1 cm, compared with the control. Isolates viz., KAL, RD, MUSH, AG and BIOC have comparatively higher starch assimilating abilities producing diameters ranging from 1.1 to 2.1 cm. Results of the starch solubilizing zones are presented in Fig. (1b).

3.4. Phosphate utilization activity

Utilization of phosphate (PO_4) in the form of di-basic (DCP) and tri-basic calcium phosphate (TCP) is demonstrated through clear zone formation around the fungal streak. Although growth was visible on Pikovskaya's agar for all the isolates, only seven isolates (viz. NIM, W1, AG, V3D, V1D, W2 and KAL) and six isolates (viz. AG, V3D, V1D, W1, KAL and NAM), are able to solubilize DCP and TCP, respectively. DCP solubilization halo zone ranges from 0.33 to 0.53 cm, with highest solubilization recorded for KAL isolate (0.053 ± 0.057). The potential of the 6 isolates (i.e. AG, V3D, V1D, W1, KAL and NAM) to solubilize TCP is lower than DCP, which ranges from 0.1 to 0.17 cm. Highest TCP solubilization of 0.17 ± 0.057 is recorded for NIM isolate. The six isolates which solubilized TCP are able to solubilize DCP, but the isolate W2 which solubilized DCP is unable to solubilize TCP. The solubilization potential of the PO_4 source for these isolates is demonstrated in Fig. (1 c, d). We observed lighter color of the isolates on Pikovskaya's agar than on PDA.

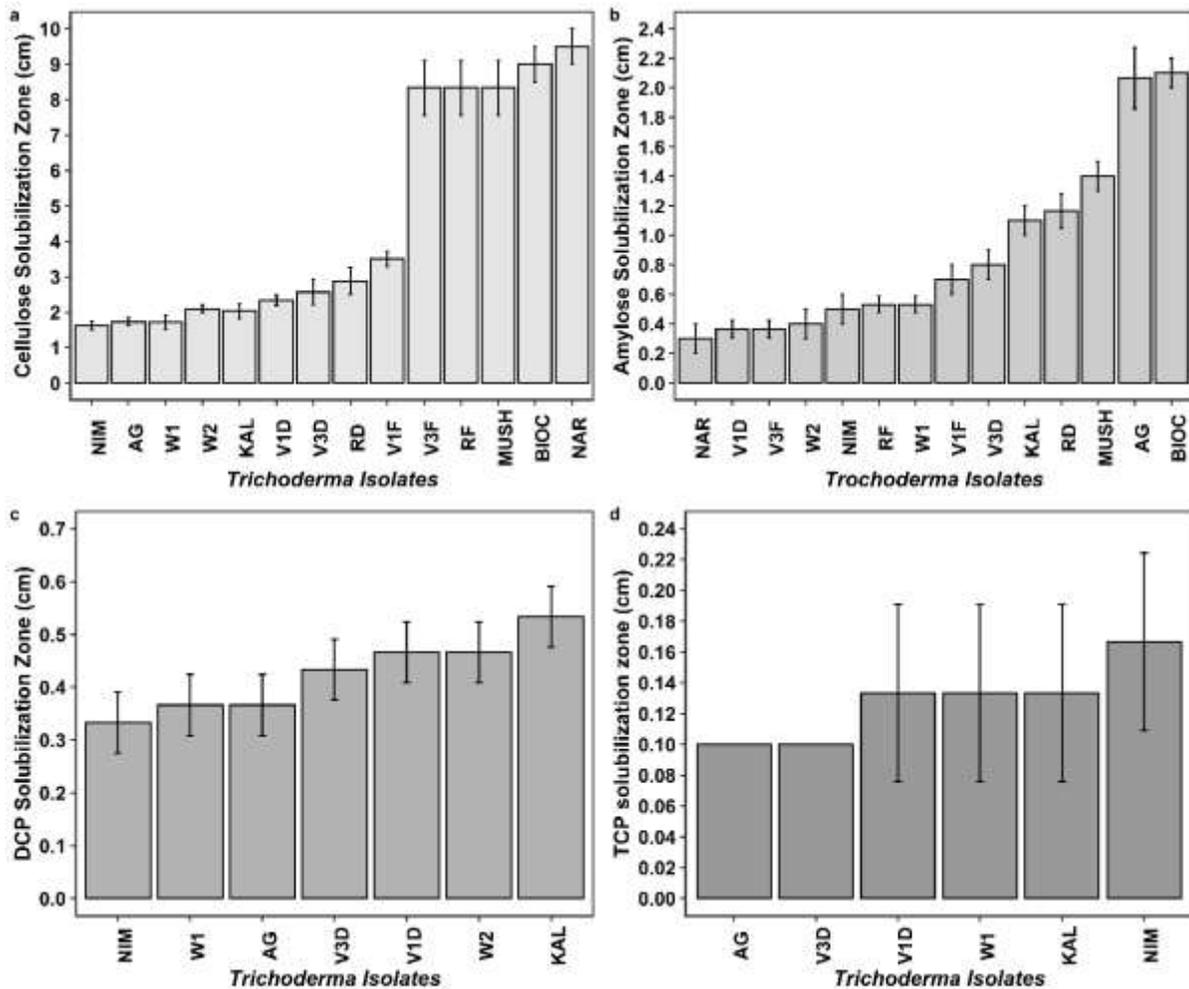


Fig. 1. Comparison of the solubilization halo zones produced by *Trichoderma* isolates on media containing; (a) Cellulose, (b) Amylose, (c) DCP and (d) TCP. Three independent experiments ($n = 3$) were conducted to access the mean. Error bars represent the standard deviation (SD).

3.5. Nitrogen assimilation potential and urease activity

All 14 *Trichoderma* isolates are able to assimilate N in the form of nitrate (KNO_3 , NaNO_3) and nitrite (NH_4NO_2) provided in the growth medium (Table 3). Radial mycelial growth and spore formation occurred on both media at similar extent, although more sporulation is observed on medium supplemented with nitrate than nitrite. Similarly, when inoculated in urea broth, all isolates except

V3F, BIOC, and MUSH are able to turn the color of the medium to bright pink, indicating the production of urease enzyme.

3.6. Biocontrol/ confrontation activity

The *in vitro* antagonistic activity of the *Trichoderma* isolates against seven plant pathogenic fungi was studied using the dual culture technique. All the fourteen isolates have some degree of *in vitro* inhibitory activities against the mycelial growth of

Table 3. Qualitative examination of nitrogen (N) assimilation and urease activity for all the 14 *Trichoderma* isolates.

Isolates	Nitrogen assimilation and urease activity test			
	Urease activity	NH ₄ NO ₂	KNO ₃	NaNO ₃
V1D	+	+	+	+
V1F	+	+	+	+
V3D	+	+	+	+
V3F	-	+	+	+
W1	+	+	+	+
W2	+	+	+	+
KAL	+	+	+	+
NAR	+	+	+	+
BIOC	-	+	+	+
AG	+	+	+	+
MUSH	-	+	+	+
RF	+	+	+	+
RD	+	+	+	+
NIM	+	+	+	+

Where; '+' denotes utilization while '-' denotes unable to utilize various nitrogen sources

R. solani, *A. niger*, *S. sclerotium*, *F. solani* and *F. oxysporum* (data not shown), but only two isolates namely BIOC and MUSH are able to inhibit *S. rolfsii* and *B. theobromae* as well, compared to the controls. The inhibition percentages of these two isolates which showed promising mycoparasitic activity along with V3F are presented in Fig. (3). Interestingly, the isolate MUSH showed significantly higher ($p < 0.05$) antagonistic potency than the BIOC and V3F against all the tested pathogenic fungi (Fig. 3) except for the *R. solani*, where BIOC and MUSH showed comparable inhibition percentages of 65 and 66.6, respectively. Similarly, the isolate BIOC is recorded to have significantly higher ($p < 0.05$) antagonistic activity than the isolate V3F against all the seven pathogenic fungi. Post treatment analysis with significant figures marked with asterisk symbol is shown in Fig. (3). MUSH and BIOC are highly effective against *F. oxysporum* with percentage inhibition of 77 % and

72 %, respectively. *Trichoderma* isolate (MUSH) presented higher mycelial growth inhibition percentage ranging from 65-77 % against the six pathogens, which is demonstrated in Fig. (2). Among the tested pathogens, *R. rolfsii* is most resistant towards *Trichoderma* isolates inhibitory potential, as MUSH and BIOC showed low inhibition percentages of 47 and 41 %, respectively.

3.7. Optimization of growth conditions

3.7.1. Growth of isolate MUSH at different pH values

Growth optimization was carried out on the indigenous *Trichoderma* isolate-MUSH, as it demonstrated the highest antifungal potency. This isolate was cultured in PDB medium having five different pH values. The growth response in terms of the dry mycelial weight was inspected after 10 d

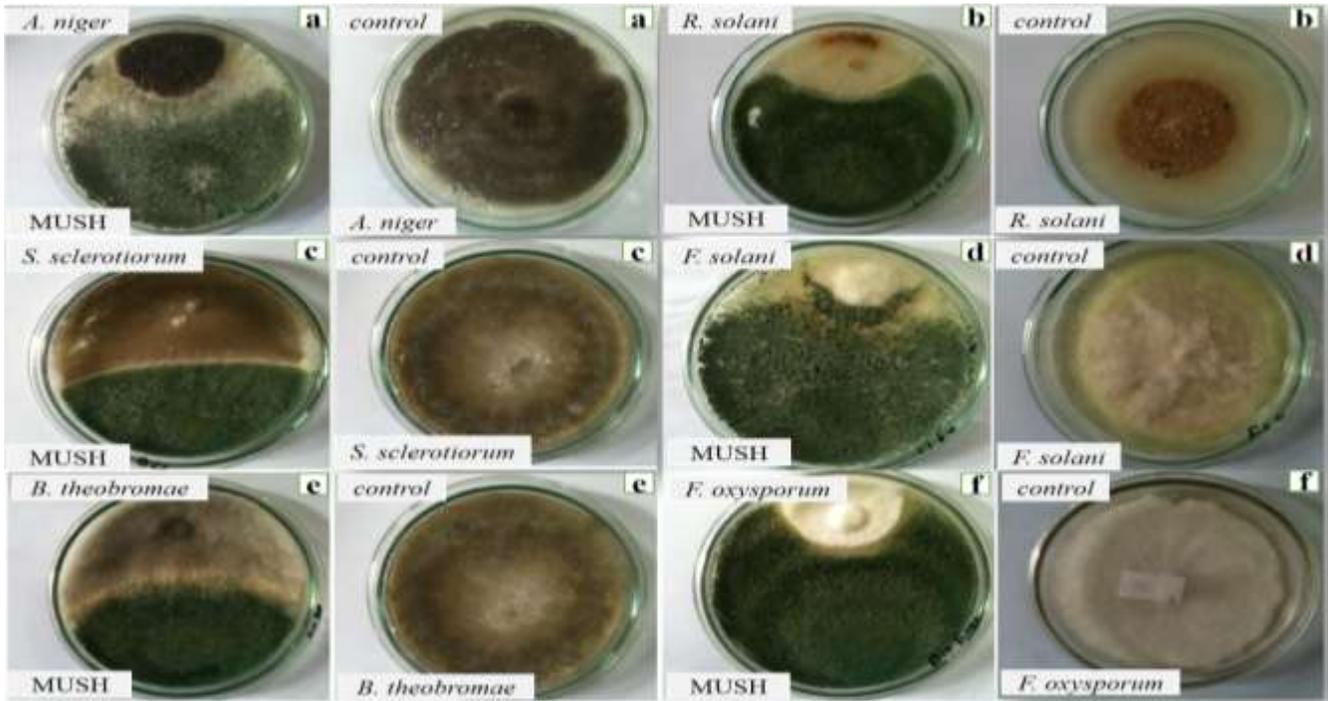


Fig. 2. Mycelial growth inhibition of (a) *A. niger*, (b) *R. solani*, (c) *S. sclerotiorum*, (d) *F. solani*, (e) *B. theobromae* and (f) *F. oxysporum* by the isolate of *Trichoderma* (MUSH) compared to their corresponding controls; using dual culture assay

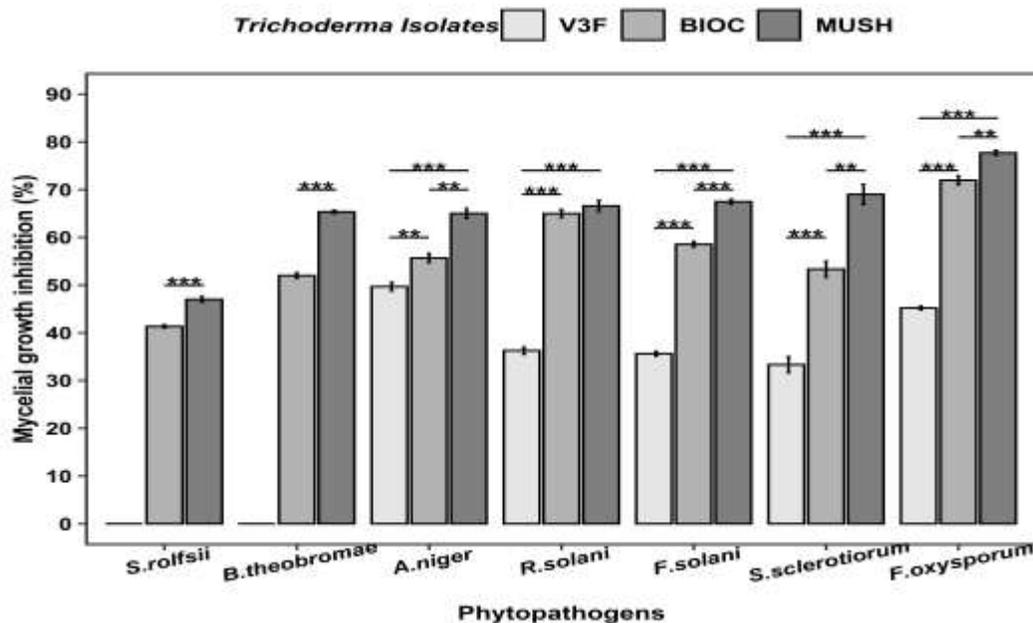


Fig. 3. Comparison of the antagonistic activity of three potent *Trichoderma* isolates against seven phytopathogens. Data from three independent experiments (n = 3) were subjected to multiple comparisons with Tukey's HSD test at $p < 0.05$. Error bars represent the standard deviation (SD). ***[$p < 0.001$], **[$p < 0.01$], *[$p < 0.05$]

of incubation. The mean recorded dry weight of isolate-MUSH (g) at pH 7 is (0.66 ± 0.05), that is significantly higher ($p < 0.01$) than in pH 5 (0.4 ± 0.09), pH 4 (0.38 ± 0.07) and pH 3 (0.37 ± 0.05), as clear in Fig (4a). The mean dry weight at pH 6 (0.55 ± 0.05) also presented significant growth promoting effect ($p < 0.05$), compared to the dry weight at pH 3 and pH 4. The dry weight of the fungal MUSH isolate at pH 3, pH 4 and pH 5 do not differ significantly ($p > 0.05$). Moreover, there is no significant difference in the mean dry weight at pH 6 and pH 7, suggesting that this pH range is the optimum required for effective growth of MUSH isolate.

3.7.2. Growth of isolate MUSH on different broth media

On PDB and at pH 7, green spores of isolate MUSH are observed after 7 d of cultivation, whereas on YMB and NB, the isolate is unable to form these green spores. The formation of mycelial mat on YMB is thicker. On NB, the mycelial mat is negligible, where only low amount of white mycelial bodies is observed. The mean fungal dry weight (g) on YMB is 2.58 ± 0.11 , which is significantly higher ($p < 0.001$) than on the PDB and NB (Fig. 4b). In addition, the recorded dry mycelial weight is also significantly higher ($p < 0.001$) on PDB (1.8 ± 0.05), compared to that on NB (0.36 ± 0.007).

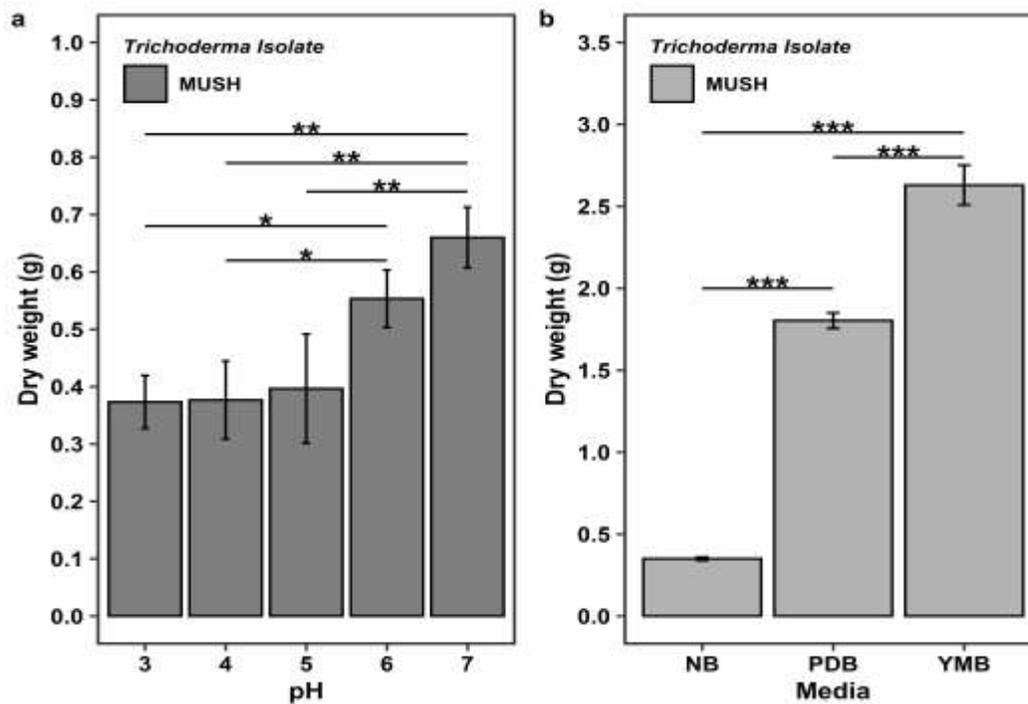


Fig 4. Comparison of dry weight of MUSH at (a) different pH levels, (b) three different synthetic broth media. Data are the mean values of three independent experiments ($n = 3$). Significantly different mean values between multiple groups were checked with Tukey's HSD post hoc treatment at $p < 0.05$. Error bars represent the standard deviation (SD). *** [$p < 0.001$], ** [$p < 0.01$], * [$p < 0.05$]

3.7.3. Growth of isolate MUSH on different solid substrates

All the grinded substrates were incubated overnight to allow water absorption. When the pH of the substrates was measured before water was drained out, it is near to the alkaline for rice husk, corn stalk and sugarcane bagasse. However, an acidic pH of 4.5 is recorded for jackfruit molasses. After incubation for 14 d, we observed abundant green mycelial mat of MUSH in the Erlenmeyer's flask containing rice husk and corn stalk, whereas comparatively less mycelial growth is detected in the flask containing jackfruit molasses and sugarcane bagasse. The rice husk and corn stalk substrates are covered with white mycelium on the 3rd day of inoculation, whereas the green conidia appeared on the 5th day of inoculation. The green conidia were counted in Neubauer chamber after extraction with Tween-80 solution. Hemocytometer count of the green conidia revealed a dense count of 72.6×10^8 cfu/ gds in corn stalk, and 68.4×10^8 cfu/ gds in the rice husk. The conidial count in sugarcane bagasse and jackfruit molasses substrates are comparatively sparse recording 12.4×10^8 and 18.6×10^8 cfu/ gds, respectively.

4. Discussion

Two species of the genus *Trichoderma* mainly *viride* and *harzianum* have great roles in the agricultural industry, due to their multiple beneficial features that promote plant growth such as; biocontrol of plant pathogens, nutrient assimilation and growth regulation (Lorito *et al.*, 2010). Considering these beneficial features, various samples were collected from different farms and substrates to screen the indigenous species of *Trichoderma*. Based on their morphological appearances, the isolates were loosely classified into two groups, either into *T. viride* or *T. harzianum*. According to Shah *et al.*, (2012), isolates producing dense green conidia on PDA are the main

characteristic features of *T. viride* and *T. harzianum*. Most of the isolates resembled *T. viride* as observed by their slender shaped phialides and globose to ellipsoidal conidia formation. Two isolates namely, MUSH and RF resembled to *T. harzianum* as observed by smooth, globose conidia and flask-shaped phialides. Large range of substrate utilization with diverse metabolism also assisted in the identification of *Trichoderma* (Kitancharoen and Hatai, 1998).

All of the recovered 14 *Trichoderma* isolates were able to utilize different forms of simple and complex sugars as seen by the appearance of yellow or orange color in media, due to the drop in pH. None isolates had the ability to hydrolyze xylan indicating their inability to produce xylanase. An early study conducted by Harman, (2000) reported that xylanase induce resistance against the plant pathogens. According to Manczinger and Pollner, (1987), simple sugars including; glucose, galactose, fructose, mannose, trehalose, xylose, arabinose and mannitol can be utilized by all *Trichoderma* strains, whereas complex carbon sources such as inulin, starch, xylan and cellulose are species specific. Qualitative analysis of the amylolytic and cellulolytic activity of the isolates showed that solubilization efficiency was higher for cellulose than for amylose. *Trichoderma* spp. are best known to produce cellulase enzyme, as highlighted by Harman, (2006). Carbohydrates that exudate from plants are hydrolyzed by the microorganisms for their nourishment, which in turn provide beneficial effects to the plants as a part of symbiosis (Druzhinina *et al.*, 2011). The cellulolytic activity can inactivate pathogen's enzymes. In accordance with the current results, Saravanakumar *et al.*, (2016) recorded that cellulase from *T. harzianum* was found to trigger induced systemic resistance (ISR) to foliar diseases in maize. Two isolates, BIOC and MUSH showed higher enzyme production in both starch and cellulose media, whereas both RF and V3F isolates showed high amylase production

activity but very low cellulase producing ability, respectively. [Mukherjee *et al.*, \(2013\)](#) revealed that the difference in enzyme production potential is largely governed by the frequency and expression level of the enzymes, in addition to the transport protein required for substrate uptake that is species specific.

Active phosphate solubilizers can be selected based on the DCP and TCP solubilization activities. Currently, the range of TCP solubilization was comparatively lower than that of DCP, which indicated that this phosphate source is harder to solubilize than DCP. [Sharma *et al.*, \(2013\)](#) documented that the phosphate group is more strongly bonded in TCP that makes it resistant to be solubilized easily. P is considered as the second most nutrient limiting factor in the agricultural soil ([Gyaneshwar *et al.*, 2002](#)). Later, a study conducted by [Saravanakumar *et al.*, \(2012\)](#) highlighted the role of *Trichoderma* spp. as effective plant growth promoting agents through solubilizing different sources of inorganic phosphates like rock phosphate, and mineralizing organic phosphate sources such as phytate, both of which are abundant in the agricultural soils. Production of organic acid was found to be the major mechanism for inorganic phosphate solubilization by fungi ([Whitelaw, 2000](#)). Generally, the P-solubilizing fungi produce more acids than bacteria, and consequently exhibit greater P-solubilizing activities ([Sharma *et al.*, 2013](#)).

All the *Trichoderma* isolates were able to utilize two nitrates (KNO₃, NaNO₃) and one nitrite (NH₄NO₂) as N sources. In accordance with the current findings, [Harman, \(2000\)](#) documented that *T. harzianum* strain T-22 increase the efficiency of using the N-containing fertilizer by maize. For three isolates viz., MUSH, BIOC and V3F, although growth was visible on urea broth, visible color change from yellow to pink was not detected. Stuart's urea broth is highly buffered that makes it insensitive to low urease production ([Stuart *et al.*, 1944](#)). The combined effect of these *Trichoderma* isolates on P and N availability to the plants provide

an effective strategy to mitigate phosphate and nitrate pollution in the ecosystem, caused by the rampant use of the chemical fertilizers. [Hoitink *et al.*, \(2006\)](#) added that the test of utilizing the different carbon, P and N sources is important, because only at the optimum nutrient sources significant population of these isolates could colonize the root rhizosphere successfully. In recent study of [Al-Hazmi and Javeed, \(2016\)](#) with *Trichoderma* spp., tomato biomass was found to be significantly higher with the increase in their inoculum size.

This study emphasized on the use of *Trichoderma* spp. as potential BCA that can provide sustainable intervention to control various plant diseases. Among the tested isolates, two isolates viz. MUSH and BIOC exhibited promising mycoparasitic activity. The two *Trichoderma* isolates (MUSH and BIOC) showed *in vitro* inhibition that ranged from 47 – 77 % and 41 - 72 %, against all the tested seven pathogens, respectively. According to [Contreras-Cornejo *et al.*, \(2016\)](#), this antifungal potential could be attributed to the production of several extracellular secondary metabolites including; viridins, gliotoxin, gliovirin, polyketides and peptaibols, in addition to the inhibitory enzymes such as serine proteases, β -glucanases, pectinases and cutinases. An *in vitro* antagonistic inhibition in the range of 4 to 100 % in dual culture was previously reported by [Kamala and Indira, \(2011\)](#); [Kotasthane *et al.*, \(2015\)](#) from indigenous *Trichoderma* isolates against different phytopathogens.

Owing to the high antagonistic potential shown by the isolate MUSH, we optimized the growth conditions such as growth at different pH and the use of several nutritional media. A previous study of [Kredics *et al.*, \(2004\)](#) revealed that the pH of medium is one of the most important environmental parameters affecting the extracellular enzyme activities associated with nutrient competition and mycoparasitism. The current study clearly recorded the inhibition of sporulation and growth of MUSH at

low pH of 3, 4 and 5. The maximum growth was found at pH 6 and 7 suggesting greater fungal growth in the soil near to the neutral rather than to acidic pH. In a previous study of [Reetha *et al.*, \(2014\)](#) with *T. harzianum*, the biomass recorded at pH 7.5 was maximum, minimum at pH 6 and very low at pH 5. At optimum pH of 7, the growth of the MUSH was studied in three synthetic broth media. After 15 d of cultivation, only a minimal dry biomass of 0.36 ± 0.007 g was obtained on NB, which may be attributed to the absence of a simple form of carbon source in NB. On the other hand, the presence of simple forms of sugars such as dextrose and mannitol in PDB and YMB media; respectively, may contribute to their significantly recorded higher biomass compared to NB. The significantly higher growth in YMB than to PDB may be attributed to the presence of different minerals including; P, Mg, Na and Ca in YMB, in addition to the presence of yeast extract which contains growth inducing factors, nitrogenous nutrients and vitamins. In accordance with the current results, previous work of [Ahamed and Vermette, \(2008\)](#) with *T. reesei* reported the higher fungal biomass and maximum production of cellulase in yeast extract medium.

An early study of [Cliquet and Scheffer, \(1997\)](#) reported that SSF on cheap available solid substrates is an attractive alternative for conidia production. This was further supported by the work of [Cavalcante *et al.*, \(2008\)](#), who found a high degree of conidiation in *Trichoderma* isolates obtained through SSF. In accordance with their findings, we recorded a significant increase in the production of green conidia by the isolate MUSH, when four locally available solid substrates were used. The density of green conidia produced in the corn stalk was high recording a count of 72.6×10^8 cfu/ gds followed by the rice husk. The low count of conidia observed on sugarcane bagasse (12.4×10^8 cfu/ gds) might be attributed to the large size of the particles. When visually inspected, both of rice husk and corn stalk were smaller in size compared to the sugarcane bagasse that have provided larger surface area for

the growth of MUSH isolate. The recorded pH in the jackfruit molasses was 4.5, which could have its negative effect on the count of conidia. In addition, different water absorption capacity and complex nutrition status might have also an impact on the conidia count. Current results showed that a conidia count in the range of 12.4×10^8 to 72.6×10^8 can be obtained from these raw substrates without any additional nutritional fortification, which warrants the possibility of fungal mass cultivation in low-cost platforms. Among rice, corn and wheat bran, a study conducted by [Cavalcante *et al.*, \(2008\)](#) recorded high conidia count on wheat bran, 28.30×10^8 cfu/ gds for *T. harzianum* and 24.30×10^8 cfu/ gds for *T. viride*, obtained after incubation for 7 d at 68 % moisture content.

Conclusion

The sustainable approach to prevent plant diseases using biocontrol activity and sufficing nutritional requirements with biofertilizers is getting huge interest as an alternative to the hazardous use of pesticides and chemical fertilizers. The *Trichoderma* spp. are considered to be important candidates and were rigorously studied for their plant growth promoting abilities. This research attempted to isolate *Trichoderma* spp. that resembled the highly active species of *harzianum* and *viride*, which can be utilized as biopesticides and biofertilizers agents. All the recovered *Trichoderma* isolates were able to utilize various sources of P, C and N, thus rendering their biofertilizer potential. Two isolates namely BIOC and V3F which resembled *T. viride* and another isolate MUSH which resembled *T. harzianum* exhibited high mycoparasitic potential against the seven tested mycopathogens. High spore count was achieved using locally available cheap substrates with SSF. This technology provides a low-cost platform for the production of BCA that can be transferred to the farmers of Nepal to produce their own plant growth promoting agents.

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Conflict of interest

No conflict of interest declared.

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Ethical Approval

Non-applicable.

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