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A modified semi-selective medium for isolation and enumeration of *Pochonia chlamydosporia* (Goddard) Zare & W. Gams

M K Rincy[1, 2], R Praveena[1*], S J Eapen[1]
[1]*Division of Crop Protection, ICAR-Indian Institute of Spices Research, Kozhikode - 673 012, Kerala, India*
[2]*University of Calicut, Malappuram - 673635, Kerala, India*
**E-mail: Praveena.R@icar.gov.in*

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Abstract

Pochonia chlamydosporia, is one of the most promising biological control agents for managing phytoparasitic nematodes. Isolation and enumeration of viable colonies of *P. chlamydosporia* from soil and other substrates without contamination is a major limitation, when commonly available nutrient media are used. Development of a suitable selective/semi-selective media by incorporating one or more inhibitors of microbial growth can facilitate isolation of the fungus. *In vitro* studies were carried out to test the compatibility of commonly used pesticides, namely, metalaxyl, metalaxyl-mancozeb, carbendazim, copper oxychloride, and chlorpyrifos with *P. chlamydosporia.* The fungus showed relatively high tolerance to higher doses of metalaxyl and carbendazim and was used in the modified medium for better suppression of other soil borne fungi. In the present study, Kerry's semi-selective medium was modified and evaluated by counting the viable fungal propagules in different substrates (rice, farmyard manure, maize, rice bran, barley, and sorghum) and soil artificially inoculated with the fungus. The results showed that the modified Kerry's semi-selective medium can effectively be used for isolation and quantification of *P. chlamydosporia* in routine studies.

Keywords: biological control, *Pochonia chlamydosporia*, quantification, semi-selective medium.

Introduction

Currently, phytoparasitic nematode management strategies are mainly based on nematicides, host plant resistance and crop rotation. Intensive use of nematicides results in critical environmental hazards (Aravind *et al.* 2009). The need for alternatives to chemical nematicides has accelerated research on eco-friendly measures to manage parasitic nematodes (Fourie *et al*. 2016). Biological control is an alternative management technique that can minimize the population of nematodes directly through parasitism or indirectly through the production of toxic metabolites and thus can address the possible environmental problems associated with chemical control of phytoparasitic nematodes (Dong & Zhang 2006).

Pochonia chlamydosporia (Goddard) Gams & Zare, a ubiquitous facultative hyperparasitic fungus, is one the most promising biocontrol agents recommended for the management of phytoparasitic nematodes. The fungus is known to infect economically significant nematode genera like *Meloidogyne*, *Globodera*, and *Heterodera* (Esteves *et al*. 2009). The fungus is a facultative parasite capable of surviving in soil as a saprotroph and parasitizing mainly the eggs and females of nematodes (Siddiqui *et al*. 2009). The fungus usually parasitizes nematode eggs without producing any specialized structures. There are various life stages of *P. chlamydosporia*, which comprise of hyphae with different nucleus numbers as well as unicellular conidiospores and multicellular chlamydospores (Kerry 2000). Chlamydospores produced by the fungus allow it to withstand challenging environmental conditions such as drought or cold. *P. chlamydosporia* strains, however, vary in their virulence, capability to colonize root surfaces, and development of chlamydospore, as well as in their effectiveness to control nematode populations (Bourne *et al*. 1994; Morton *et al.* 2003; Mauchline *et al*. 2004). To understand the efficiency of *P. chlamydosporia,* it is important to monitor the growth, establishment and survival in the soil and on plant roots. Quantification of *P. chlamydosporia* is a limitation, since the fungus does not consist of propagules that are of same size and genetic material (Manzanilla-lópez *et al*. 2011)**.** Besides, quantitative estimation of the fungal population from rhizosphere soil samples is often difficult because of the slow growing nature of the fungus on conventional agar media.

Isolation and counting of *P. chlamydosporia* colonies will be much easier and faster if a suitable semi-selective media is developed by incorporating one or more inhibitors of microbial growth. The usage of semi-selective media for isolation and enumeration can restrict bacterial contamination and fast-growing fungal colonies, and can also aid in determining the comparative abundance of fungus in the root rhizosphere infected with nematodes (Mauchline *et al.* 2002; Atkins *et al.* 2003a). Enumeration of *P. chlamydosporia* from soil and other substrates using Kerry's semi-selective medium results in the contamination of the medium by colonies of other soil borne fungi. The present study was therefore carried out to develop a modified semi-selective medium for isolation and quantification of the fungus from humid tropical soils and other substrates.

Materials and Methods

Culture characteristics of P. chlamydosporia (NAIMCC-SF-0048)

The isolate used in the present study was *P. chlamydosporia* (NAIMCC-SF-0048) maintained in the repository for biocontrol agents at ICAR-Indian Institute of Spices Research (IISR), Kozhikode, India. The identity of the strain was confirmed by DNA fingerprinting at National Buraeu of Agriculturally Important Microorganisms (NBAIM), Mau, Uttar Pradesh.

To study the culture characteristics, mycelial discs of size 5 mm (diameter), taken from the edges of the *P. chlamydosporia* colony were placed in the centre of the potato dextrose agar (PDA) plates and incubated in triplicate at 28± 2° C, 70- 80% RH and a 12:12 h day: night photo phase. The mean radial mycelial growth (mm), colony size and colour were recorded 10 days after inoculation (DAI). The experiment was repeated once to confirm the results.

Conidial morphology was studied by mounting the 14 day old fungal culture in lactophenol on microscopic slides and the size of conidia was measured at 10x and 40x magnification under a bright field microscope (Leica DM5000B). The morphological characters were compared with the descriptions given in the published keys of the fungal species (Sung *et al*. 2007; Atkins *et al*. 2003b).

In vitro compatibility of P. chlamydosporia with pesticides

Five pesticides *viz*., carbendazim (Bavistin 50% WP), metalaxyl-mancozeb (Master 72% WP), metalaxyl (Metalaxyl 35% WP), copper oxychloride (Blitox 50% WP), and

chlorpyrifos (Anth 50% EC) were tested for their compatibility with *P. chlamydosporia* by poisoned food technique (Bruin *et al.* 1981) at the recommended dose, two lower doses and two higher doses as given in Table 1. Stock solution of each pesticide was prepared by adding the requisite quantity of the chemical in sterile distilled water and added to molten PDA in Erlenmeyer flasks separately to get the final concentrations. The poisoned medium was poured into sterile Petri dishes of 9 cm diameter and allowed to cool. Mycelial discs of size 5 mm diameter taken from the edge of *P. chlamydosporia* colony was placed in the centre of each Petridish and incubated for 10 days as described above. Petri dishes with non-poisoned medium served as the control. Each treatment was replicated thrice. The radial growth of the colony was measured in each treatment and the percentage inhibition of growth was determined using the formula: I = C - T/C x 100 where, I is percentage growth inhibition, C is radial growth in control (mm) and T is radial growth in treated plates (mm). The tolerance limit of pesticide concentration for the fungus was worked out and values were statistically analysed.

Modification of Kerry's semi-selective medium

The Kerry's semi-selective medium (Kerry *et al.* 1993) is as follows: 17 g CMA, 17.5 g NaCl, 75 mg Rose Bengal, 37.5 mg of carbendazim and thiabendazole, 50 mg each of the antibiotics chloramphenicol, aureomycin and streptomycin sulphate and 3 ml Triton X-100 per litre. In the present study, the semi-selective medium was modified with the following composition: 17 g corn meal agar, 17.5 g NaCl, 75 mg Rose Bengal, 50 mg each of metalaxyl and carbendazim, 3 mL Triton X-100, 50 mg each of rifampicin, streptomycin sulphate and chloramphenicol per litre.

Evaluation of modified semi selective media

The modified semi selective media was evaluated by dilution-plate technique. Serial dilutions were prepared from 1 ml liquid inoculum of *P. chlamydosporia* grown in potato dextrose broth (PDB) as described above. Aliquots (0.2 ml) of each dilution in triplicate were aseptically spread on the surface of the selective media in Petri dishes (9 cm) and the plates were incubated as described above for 10 days. The colonies of serially diluted plates were counted and expressed in terms of colony forming units (CFU) mL^{-1}.

Isolation and quantification of P. chlamydosporia from different solid substrates and soil

The efficiency of the modified Kerry's semi-selective medium was studied using different solid substrates namely, sorghum, rice, barley, rice bran, maize, farmyard manure, and soil artificially inoculated with *P. chlamydosporia*. For this, 40 g each of sorghum, rice, barley, rice bran, and maize were soaked separately in distilled water for 1-2 hours. The soaked grains were washed with distilled water and the substrates were autoclaved at 15 psi for 20 minutes. To inoculate the solid substrates, liquid inoculum of *P. chlamydosporia* was prepared by inoculating PDB in Erlenmeyer flasks with mycelial discs of size 5 mm taken from the margins of the colony (5 discs 250 ml^{-1} media). The flasks were kept inside an incubator shaker maintained at 28 ± 2° C, 180 rpm for 10 days.

Table1. Pesticides and concentrations used for *in vitro* compatibility studies with *Pochonia chlamydosporia*

Pesticide	Concentrations tested (ppm)				
	1/4x	1/2x	x	2x	4x
Metalaxyl	250	500	1000	2000	4000
Carbendazim	250	500	1000	2000	4000
Metalaxyl - mancozeb	312.5	625	1250	2500	5000
Copper oxychloride	500	1000	2000	4000	8000
Chlorpyriphos	750	1500	3000	6000	12000

After sterilization, each flask with the solid substrates was inoculated with 10 ml of the liquid fungal inoculum. The inoculated flasks were shaken at regular interval to obtain uniform growth of the fungus. The assays were carried out in triplicates and the experiment was repeated three times to confirm the results. *P. chlamydosporia* on different solid substrates and soil was quantified 14 days after inoculation. The colonies were counted and expressed in terms of CFU g^{-1} of the substrate.

Isolation and quantification of P. chlamydosporia from in planta studies

A pot experiment was set to test the efficacy of the developed medium for enumerating the colonies of *P. chlamydosporia* in soils under greenhouse conditions (28 ± 2°C, relative humidity 70-80%. One month old black pepper plants (Variety: IISR Sreekara) were planted in pots filled with potting mixture (1 kg) like sterilized soil, non-sterilized soil and vermiculite + farmyard manure. Inoculum of *P. chlamydosporia* (10^8 cfu g^{-1}) mass multiplied on rice grains were applied to the planting media at different doses (1 g, 3 g and 5 g) one month after planting. All the treatments were replicated thrice and control plants were maintained without inoculating the fungus. Soil sampling was done 1 month after post inoculation, serially diluted and plated in semi-selective medium and incubated at 28 ± 2°C, 70-80% RH for 10 days. The colonies of serially diluted plates were counted and expressed in terms of CFU mL^{-1}.

Results and Discussion

The isolate of *P. chlamydosporia* used in the present study produced white cottony mycelium, with a light-yellow centre and even edges. The colony diameter ranged between 2.5 - 3.0 cm on PDA, seven days after inoculation indicating the slow growing nature of the fungus. Microscopic observation showed that the conidia were produced individually on vegetative hyphae or in two whorls on erect conidiophores. The mycelium was verticillate in nature and the conidia produced were ellipsoid in shape. The morphological characters of the isolate used in the study were in confirmation with the descriptions given by Zare *et al*. (2001). Based on the fingerprinting data of NBAIM Mau, the fungal isolate was confirmed as *P. chlamydosporia,* with the accession number NAIMCC-SF-0048. According to Zare *et al*. (2001), conidial characters are mainly used to identify the variety of *P. chlamydosporia*. The isolates of *P. chlamydosporia* var. *catenulate* produced conidia which are more globose to subglobose in shape whereas, *P. chlamydosporia* var. *chlamydosporia* produced ellipsoid conidia.

Due to the slow growing nature of the fungus on culture media, quantitative estimation of the population in soil and other substrates is a major limitation. For the easy isolation and identification of *P. chlamydosporia,* the use of an appropriate semi-selective media with microbial inhibitors is essential. Use of semi selective media can prevent the growth of bacteria and other fungi and also help to estimate the population of the fungus in the rhizosphere of plants (Mauchline *et al.* 2002; Atkins *et al.* 2003a). In the present study, when Kerry's semi-selective medium was used for soil population estimation, the inoculated plates got contaminated with the colonies of other soil inhabiting fungi *viz*., *Pythium*, *Fusarium* etc. Hence attempts were made to amend the above medium so that isolation and diagnosis of the target fungus from humid tropical soils and other substrates was easy and accurate.

The *in vitro* compatibility studies of *P. chlamydosporia* with the five pesticides *viz*., carbendazim, metalaxyl- mancozeb, metalaxyl, copper oxychloride, and chlorpyrifos clearly showed that the fungus could withstand high concentrations of metalaxyl (0.4%) and carbendazim when compared to other pesticides (Table 2). The effective dose concentrations (ED50) of metalaxyl and carbendazim were found to be 2.36% and 1.32%, respectively. *In vitro* effects of different concentrations of metalaxyl and carbendazim on mycelial growth of *P. chlamydosporia* are shown in Figures 1 and 2, respectively. Copper oxychloride, the combination product of

Table 2. Compatibility of *Pochonia chlamydosporia* with different pesticides

Pesticides	Concentration (ppm)	Percentage growth inhibition (I) (%)*	ED_{50}
Metalaxyl	250	4.76 ± 0.017^{D}	2.36
	500	9.52 ± 0.011^{C}	
	1000	9.52 ± 0.029^{C}	
	2000	19.04 ± 0.023^{B}	
	4000	23.80 ± 0.005^{A}	
Carbendazim	250	33.3 ± 0.034^{B}	1.32
	500	33.3 ± 0.040^{B}	
	1000	42.85 ± 0.023^{A}	
	2000	42.85 ± 0.017^{A}	
	4000	42.85 ± 0.028^{A}	
Metalaxyl - Mz	312.5	42.85 ± 0.046^{C}	0.059
	625	42.85 ± 0.080^{C}	
	1250	52.38 ± 0.046^{B}	
	2500	100 ± 0^{A}	
	5000	100 ± 0^{A}	
Copper oxychloride	500	33.3 ± 0.069^{B}	0.051
	1000	33.3 ± 0.086^{B}	
	2000	100 ± 0^{A}	
	4000	100 ± 0^{A}	
	8000	100 ± 0^{A}	
Chlorpyriphos	750	42.8 ± 0.057^{C}	0.11
	1500	47.6 ± 0.029^{B}	
	3000	100 ± 0^{A}	
	6000	100 ± 0^{A}	
	12000	100 ± 0^{A}	

* Values are means of three replicates ± standard error (S.E). I = C - T/C x 100 where, I = Percentage growth inhibition; C = Radial growth in control (mm); T = Radial growth in treated plates (mm), ED_{50} (Effective dose). *Means followed by the same letter are not statistically different.

metalaxyl – mancozeb, and chlorpyriphos inhibited the fungus at concentrations above 2000 ppm. Based on the *in vitro* evaluation, the fungicides metalaxyl and carbendazim were selected to modify Kerry's semi-selective medium. The compatibility studies of 14 agropesticides on the development and sporulation of nematophagous fungi showed that the isolates of *Paecilomyces* sp. and *Pochonia* sp. were less sensitive to the pesticides tested than *Arthrobotrys* species (Mensin*et al.* 2013). Jacobs *et al.* (2003) reported that the nematophagous fungi such as *P. lilacinus, Plectosphaerella cucumerina,* and *P. chlamydosporia* were compatible with the chemical tolclofos-methyl.

Based on the leads obtained in the present study, a modified semi-selective medium with the following composition was prepared as explained in materials and methods. The fungicides and antibiotics used in the medium can inhibit the growth of other fungi and

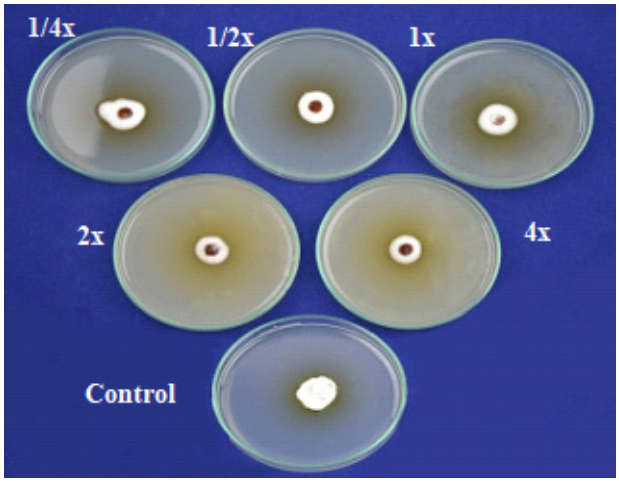

Fig 1. *In vitro* evaluation of different concentrations of metalaxyl on mycelial growth of *Pochonia chlamydosporia* (1/4x- 250 ppm, 1/2x-500 ppm, 1x-1000 ppm, 2x- 2000 ppm, 4x- 4000 ppm)

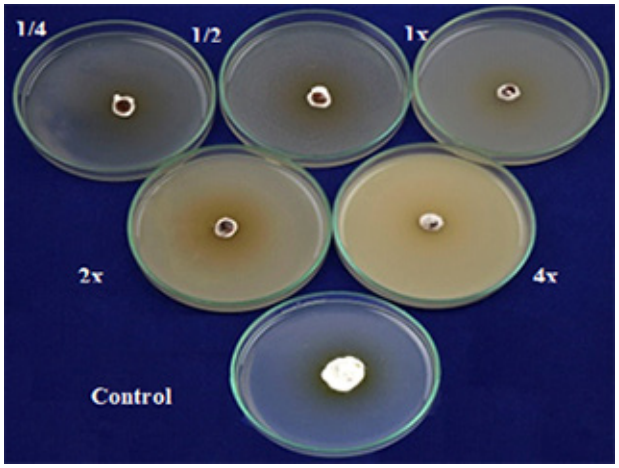

Fig 2. *In vitro* evaluation of different concentrations of carbendazim on mycelial growth of *Pochonia chlamydosporia* (1/4x- 250 ppm, 1/2x-500 ppm, 1x-1000, 2x- 2000 ppm, 4x- 4000 ppm)

bacteria, respectively. NaCl and Triton X-100 reduced the rate of colony growth and the Rose Bengal also prevented the growth of microbes. Manzanilla-lópez *et al*. (2017) reported that the microbial contamination in media can affect the isolation of nematophagous fungi. The use of fungicides metalaxyl and carbendazim in the modified media prevented the fast-growing fungi like *Pythium* and *Fusarium* species, respectively, and the identification and enumeration of *P. chlamydosporia* colonies were comparatively easier on this modified medium. The reddish yellow colonies of *P. chlamydosporia* obtained after serial dilution in modified semi-selective media are shown in Figure 3. The colonies formed on semi-selective media were reinoculated on potato dextrose agar to reconfirm the colony characters. The reinoculated colonies showed the morphological characters of the test isolate used for the study.

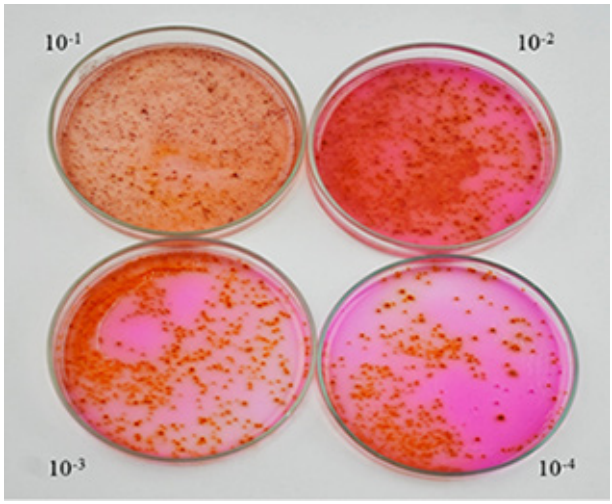

Fig 3. *Pochonia chlamydosporia* colonies in modified semi-selective medium after serial dilution.

Growth of *P. chlamydosporia* on various solid substrates *viz*. sorghum, rice, barley, rice bran, maize, farmyard manure, was enumerated using the modified medium (Figure 4). Among the substrates tested, highest CFU g^{-1} was observed with rice grains; the population recorded was significantly higher compared to other substrates tested. Hidalgo *et al.* (2000) reported that rice can be used as a substrate for the mass production of *P. chlamydosporia*. Under *in vitro* conditions, isolation and quantification of colonies of *P. chlamydosporia* from various substrates using the modified medium were easier, faster and without any bacterial and fungal contamination.

Growth of *P. chlamydosporia* in soil inoculated

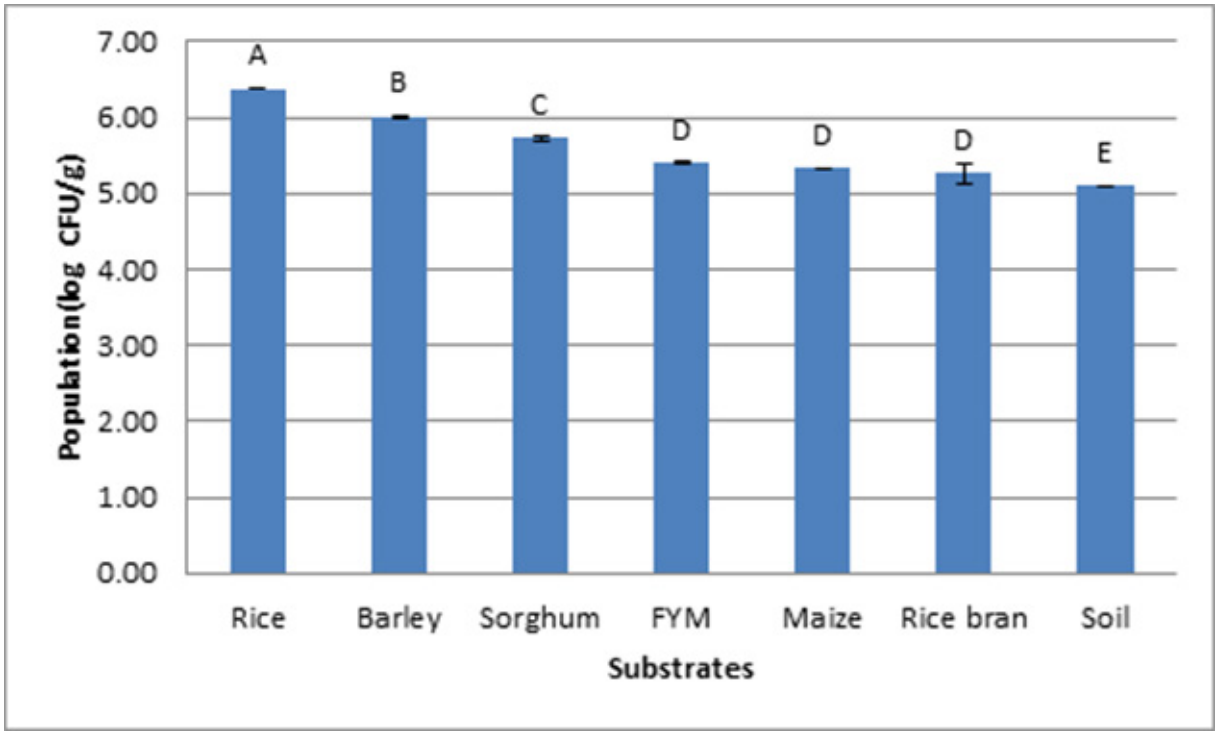

Fig 4. Quantification of *Pochonia chlamydosporia* on different solid substrates and soil 7 days after inoculation.

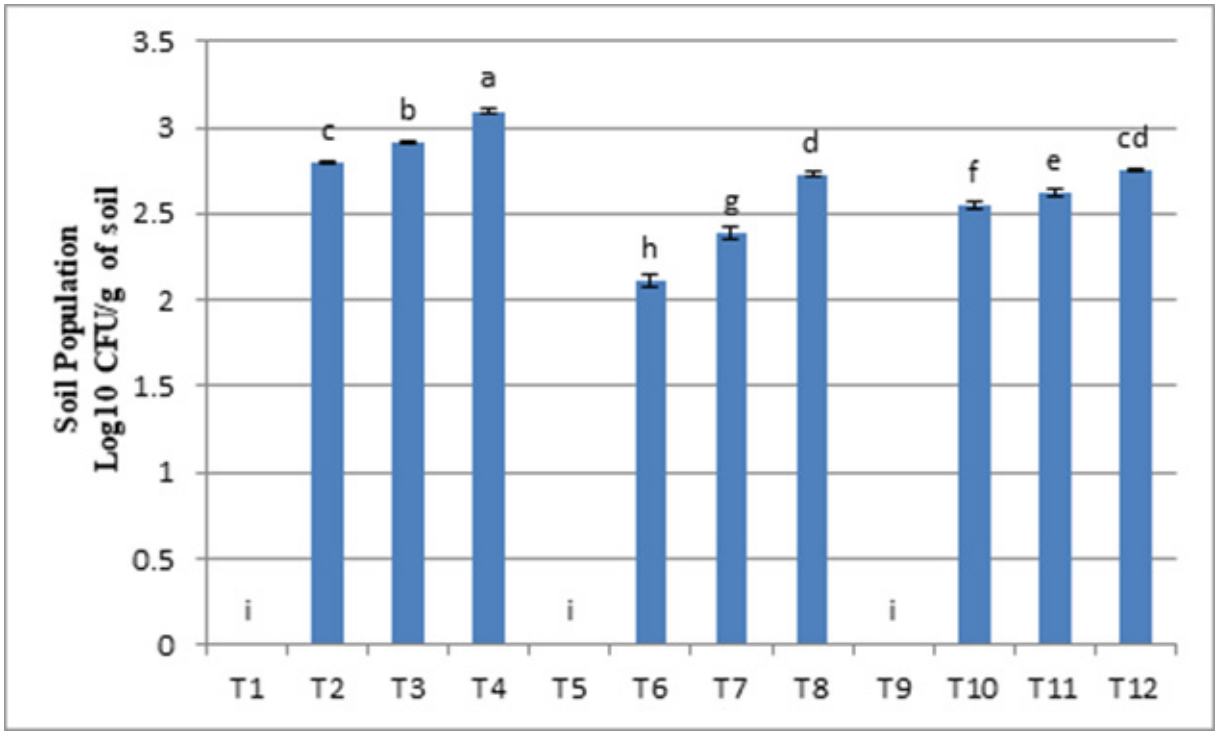

Fig 5. Quantification of *Pochonia chlamydosporia* (10^8 cfu g^{-1}) mass multiplied on rice grains in potting mixture 30 days after inoculation.

T_1: Uninoculated sterile soil, T_2: Sterile soil inoculated with 1 g* T_3: Sterile soil inoculated with 3 g, T_4: Sterile soil inoculated with 5 g, T_5: Uninoculated non-sterile soil, T_6: Non sterile soil inoculated with 1 g, T_7: Non sterile soil inoculated with 3 g, T_8: Non sterile soil inoculated with 5 g, T_9: Uninoculated vermiculite + farmyard manure, T_{10}: Vermiculite + farmyard manure inoculated with 1 g, T_{11}: Vermiculite + farmyard manure inoculated with 3 g, T_{12}: Vermiculite + farmyard manure inoculated with 5 g.

with different doses of the fungus was also enumerated using the modified medium and the results are presented in Figure 5. The potting mixture inoculated with the fungus recorded significantly higher population one month after inoculation. The modified selective media was used for enumeration o and the medium was useful in estimating the population of *P. chlamydosporia* from non sterile soil samples without any fungal or bacterial contamination. The population of *P. chlamydosporia* was significantly high in treatments with soil than vermiculite + farmyard mixture. The population of *P. chlamydosporia* was found maximum in sterile soil inoculated with 5 g of inoculum.

The media developed was also useful in estimating the population of *P. chlamydosporia* isolated from rhizosphere soil samples without any contamination. In the present study, Kerry's semi-selective medium was modified for the efficient isolation and quantification of *P. chlamydosporia* from soils and other mass production substrates.

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