Short Communication

Low cost carrier material for mass production of *Trichoderma* inoculants

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Abstract

A study was conducted to explore the possibility of solid state fermentation technique for mass production of *Trichoderma viride* using sterilized rice powder. Sterilized rice powder was supplemented with dextrose (3%) and the population reached $123x10^{15}$ cfu g^{-1} at 10^{th} day and the inoculum could be stored upto 180 days with a population of $10x10^{9}$ cfu g^{-1} under room temperature. The viability increased ($28x10^{12}$ cfu g^{-1}) when it was stored at refrigerated condition. The spore biomass produced could be either used directly for seed treatment, seed biopriming, soil/root treatment, wound dressing etc., or for formulation. Talc with rice powder+dextrose based inoculum @ 2% along with 8% sterile water sustained the viability of $6x10^{8}$ cfu g^{-1} at 180 days and could be a feasible method for large scale inoculum production of fungal biocontrol agents.

Key words: Formulation, Rice powder and Dextrose, Shelf life, Solid state fermentation, *Trichoderma viride*.

Trichoderma spp. has gained significant scientific attention as a potential biocontrol agent for the management of several soil borne diseases. A number of *Trichoderma* based products have been commercialized in India and abroad. For industry based large scale production, liquid state fermentation technology has been employed while solid state fermentation technology has been in use for on-farm production or small scale production (Ramanujam et al., 2010). In large scale production, *Trichoderma* inoculum is generally multiplied in liquid media like molasses – yeast or potato dextrose broth and mixed in inert carrier materials (Prasad and Rangeshwaran, 1998). However, in this process, it is experienced that the viability of inoculum in liquid media reduced drastically within a few weeks (Papavizas et al., 1984), and hence the inoculum could not be stored for longer periods. Production of large quantities of good quality inoculum with longer shelf life which can be formulated with any carrier material is an essential requirement for meeting the escalating demand of this biocontrol agent. Compared to the submerged (liquid state) fermentation technology, solid state fermentation has several advantages such as easiness in preparation, high productivity, low cost equipments, lesser waste generation and lesser time and energy requirement (Kocher et al., 2008). Several organic substrates including grains like sorghum, wheat, maize, rice etc. depending upon local availability have been utilized for mass cultivation of *Trichoderma* inoculum (Rini and Sulochana, 2007; Mridula et al., 2012; Kishore et al., 2014). Hence, the present study was taken up to explore the feasibility of utilizing solid state fermentation technology using sterilized rice grain

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powder for mass production of *Trichoderma* inoculum and to assess the feasibility of its use in large scale production programmes.

Rice grains (raw rice/pachari) were made soft by soaking in water (1:2 w/v) for 30 minutes and the excess water was removed by transferring the soaked grains into a strainer vessel. The grains were then powdered to a coarse texture using a mixer grinder. Dextrose (3%) was added as nutritional supplement for enhancing the conidial yield. Powdered rice grain (100g) was taken in 250 ml conical flasks, plugged, sterilized at 1.02 kg cm^{-2} for 20 minutes and inoculated aseptically with 2-3 culture discs (1 cm^2) of actively growing *Trichoderma viride* (Kerala Agricultural University isolate) or 0.5 g of previously prepared rice powder based *Trichoderma* inoculum. The contents were then incubated at room temperature (28±2°C) for seven days after which it was transferred and spread in clean plastic trays. The trays were covered with blotting paper or ordinary newspaper and kept for two more days for maximum sporulation. This process was done in a non-sterile room condition. Visual observations on growth of *T.viride* were recorded daily. The number of days taken by the fungus for complete growth, and sporulation over the substrate was recorded. The sporulated fungus was inoculated on sterile potato dextrose agar (PDA) media and its identity was confirmed based on cultural and morphological characters (Barnett and Barry, 1972). The green spore mass was then sieved and stored (50 g/pack) in polythene packets with and without refrigeration. One gram sample was aseptically drawn from the packets and population assessed following serial dilution plate technique (Seeley and VanDemark, 1981) using PDA supplemented with Rosebengal @ 25 mg L^{-1} at 30 days interval. The number of fungal colonies were counted after two days of incubation and expressed as colony forming units (CFU) per gram of substrate. Three samples were maintained in each step. Simultaneously, the spore mass was formulated in talc (commercial grade, non sterilized) with four different concentrations *i.e.,* 1, 2, 5 and 10% and the population assessed at fortnightly intervals till 90 days and thereafter at 30 days interval. The inoculum concentration which supported maximum propagule density with prolonged shelf life was further evaluated for the effect of moisture on spore viability. Talc was formulated with rice powder based *Trichoderma* inoculum at selected concentration. The moisture level was enhanced by adding sterile water at 8%. Talc was also mixed with potato dextrose broth based *Trichoderma* inoculum (250 ml/kg talc). Population estimation was done on the first day and repeated at fifteen days interval till 90 days, and then at 30 days interval upto 180 days with PDA-Rose Bengal medium using dilution plate technique. The data were subjected to Analysis of Variance technique using Web Agri Stat Package (WASP 2.0).

It was observed that white mycelia of *T. viride* grew completely into the rice powder within five days and green sporulation covered the entire media in seven days (Fig.1). The whole mass of media overgrown by *Trichoderma* was then emptied from the conical flasks, transferred, spread in clean plastic trays and covered. In trays, complete sporulation occurred and the entire media changed into dark green spore mass in another two days (Fig.2). *T. viride* was able to multiply in sterilized rice powder + dextrose (3%) and a very high population ($123x10^{15}$ cfu g^{-1}) was achieved at 10^{th} day of

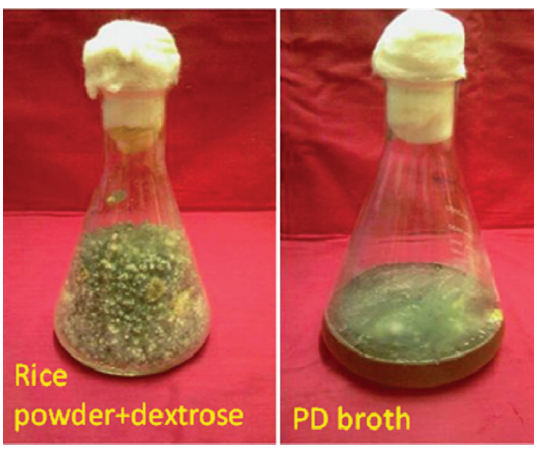

Figure1. Growth of *T. viride* spore mass in rice powder+dextrose and potato dextrose broth

Figure 2. Rice powder+dextrose based *T. viride* spore mass

inoculation (Table 1). *Trichoderma* has the potential to reach vey high population in various organic substrates. Nakkeeran et al. (2005) produced *T. viride* on sorghum grains and achieved a population of $1x10^{11}$ cfu g^{-1}. Sayit et al. (2013) reported that after 4 days of cultivation under optimum conditions, *T. harzianum* could reach a population of $1.30 \pm 0.68 \times 10^{10}$ cfu g^{-1} dry substrate of wheat bran–malt sprout mixture. Hence, our results are also in accordance with the above reports. The easy availability of nutrients from the powdered rice and dextrose might have resulted in the very high population of *T. viride* in our study.

Though a gradual decline in population was recorded during storage, substantially good quantity of viable propagules ($10x10^9$ cfu g^{-1}) could be maintained even at 180 days or six months of storage under room temperature. Under refrigerated condition at 4°C, the population did not reduce significantly till 30 days and thereafter declined gradually. It is clear that the viability of propagules was more in refrigerated condition at 4°C, as evidenced by $28x10^{12}$ cfu g^{-1} at 180 days. Interestingly, in this process, though the contents were kept in non sterile room condition for sporulation, there were no contaminating organisms other than *T. viride* in the culture plates during plating. Contaminants in the substrate might have been hindered by the antagonistic nature of *T. viride* in the media. In the case of submerged inoculum in potato dextrose broth, though $31x10^{15}$ cfu ml^{-1} was recorded at 10th day, a steady decline in the viability occurred at 30 days ($6x10^6$ cfu ml^{-1}) beyond which the population was not assessed. Potato dextrose broth was reported to be one of the best media for biomass production of *T. harzianum* (Ferdous et al., 2016). Sally et al.(2010) reported a population of $364 x10^9$ cfu ml^{-1} of *T. harzianum* in potato dextrose broth on 10th day of inoculation. In the present study,

Table 1. Population of *Trichoderma viride* in rice powder+dextrose and potato dextrose broth

Days after inoculation 10 (Pre storage)	Mean population (cfu g^{-1}sample)*		
	Rice powder+dextrose based *T. viride* inoculum 123 x 10^{15} (17.09)[a]		*T. viride* inoculum in potato dextrose broth 31 x 10^{15} (16.47)[a]
	At room temperature (28±2°C)	At refrigerated condition (4°C)	At room temperature (28±2°C)
30	51 x 10^{15} (16.70)[b]	97 x 10^{15} (16.98)[a]	6 x 10^6 (6.76)[b]
60	12 x 10^{14} (15.06)[c]	53 x 10^{15} (16.72)[b]	-
90	23 x 10^{10} (11.36)[d]	14 x 10^{15} (16.12)[c]	-
120	10 x 10^{10} (10.96)[e]	26 x 10^{14} (15.41)[d]	-
150	20 x 10^9 (10.30)[f]	31 x 10^{13} (14.49)[e]	-
180	10 x 10^9 (9.99)[g]	28 x 10^{12} (13.44)[f]	-
CD (0.05%)	0.226	0.169	0.338

* Mean of three replications

Figures in parentheses are log transformed values

a very high population of *Trichoderma* was obtained when compared to the above result. Gao (2016) stated that biomass yields and sporulation of this fungus depends on the culture conditions, nutritional requirements including carbon and nitrogen source, mineral elements, carbon concentration, carbon to nitrogen ratio, together with environmental factors like water potential, pH, dark/light cycle and temperature. The very high population density obtained in our study may be attributed to the variation in any of the above conditions. At 30 days, the population of *Trichoderma* in potato dextrose broth declined drastically. The limitation of available nutrients in the media due to the very high population might have accelerated the loss in viability of *Trichoderma* at 30 days of incubation.

Among the different concentrations *viz*., 1, 2, 5 and 10 % tested for formulation, addition of 2% sterilized rice powder+dextrose based inoculum in talc sustained equal number of viable propagules as that of 5% and 10% concentration at 180 days. Hence, formulation @ 2% was selected for studying the effect of moisture on spore viability. In talc mixed with 2% rice powder+dextrose based *Trichoderma* inoculum, the initial population was $18x10^{12}$ cfu g^{-1}. Though the population started declining from 15 days, a high number of viable propagules was maintained till 90 days ($2x10^{10}$ cfu g^{-1}) and thereafter declined steadily. Whereas in talc mixed with 2% rice powder+dextrose based *Trichoderma* inoculum and 8% sterile water, the population ($26x10^{12}$ cfu g^{-1}) increased at 15 days, remained the same till 30 days and gradually declined. However, augmenting moisture level by 8% sustained the viability of the organism at high proportions upto 180 days. At 180 days, the population recorded in formulation without any added moisture was $1x10^{6}$ cfu g^{-1} while $6x10^{8}$ cfu g^{-1} was maintained in formulation supplied with 8% sterile water (Table 2). When talc was formulated using submerged *Trichoderma* culture (250 ml/kg talc), the initial population recorded was $5x10^{10}$ cfu g^{-1}. The population showed an increasing pattern till 30 days ($30x10^{10}$ cfu g^{-1}) and then decreased gradually to $2x10^{6}$ cfu g^{-1} at 180 days. From the data, it is evident that formulating talc with 2% rice powder+dextrose based *Trichoderma* inoculum along with 8% sterile water could sustain more number of viable propagules at 180 days when compared to the formulation using submerged *Trichoderma* culture in potato dextrose broth. Papavizas et al. (1984) reported that the propagules of *Trichoderma* produced in liquid fermentation have thinner cell walls and may have lesser desiccation tolerance and shelf life. According to

Table 2. Population of *Trichoderma viride* in talc formulation using rice powder+dextrose and potato dextrose broth based inoculum

Days after inoculation	Mean population of *T. viride* (cfu g^{-1} sample) *		
	Talc + Rice powder-dextrose based*Trichoderma* inoculum (2%)	Talc + Rice powder-dextrose based *Trichoderma* inoculum (2%) + 8% sterile water	Talc + Potato dextrose broth based *Trichoderma* @ 25%
0	18×10^{12} (13.25)^{a}	26×10^{12} (13.41)^{ab}	5×10^{10} (10.62)^{bc}
15	17×10^{10} (11.22)^{b}	35×10^{12} (13.54)^{a}	7×10^{10} (10.84)^{b}
30	3×10^{10} (10.48)^{c}	31×10^{12} (13.48)^{a}	30×10^{10} (11.47)^{a}
45	3×10^{10} (10.47)^{c}	22×10^{12} (13.34)^{ab}	4×10^{10} (10.60)^{bc}
60	3×10^{10} (10.47)^{c}	16×10^{12} (13.20)^{b}	3×10^{10} (10.46)^{c}
75	2×10^{10} (10.3)^{d}	9×10^{12} (12.95)^{c}	3×10^{10} (10.39)^{c}
90	2×10^{10} (10.30)^{d}	14×10^{10} (11.15)^{d}	1×10^{10} (9.99)^{d}
120	3×10^{7} (7.48)^{e}	8×10^{10} (10.89)^{e}	3×10^{8} (8.40)^{e}
150	2×10^{6} (6.29)^{f}	1×10^{10} (9.96)^{f}	1×10^{8} (7.99)^{f}
180	1×10^{6} (5.30)^{g}	6×10^{8} (8.73)^{g}	2×10^{6} (6.30)^{g}
CD (0.05%)	0.129	0.232	0.348

*Mean of three replications

Figures in parentheses are log transformed values

Lumsden et al. (1995) a formulated biocontrol product with agricultural application should possess abundant viable propagules with good shelf life.

In India, talc based formulation of *Trichoderma* is quite popular and several small and large entrepreneurs produce large quantities of talc formulations for supply to farmers. According to Jeyarajan (2006), the annual requirement of *Trichoderma* has been estimated as 5,000 tonnes to cover 50 per cent area in India. The National Farmer Policy (2007) has strongly insisted on the promotion of biopesticides for sustainable agricultural production. However the share of biopesticides in India is merely 2% (Sabalpara, 2014). To meet the massive requirement of *Trichoderma* in India, enormous quantity of inoculum is required. Sanjeev et al. (2014) opined that standardizing formulation of superior strains which supports increased shelf life with low level of contaminants is required for making biocontrol a successful commercial venture. They also stated that solid state fermentation is an effective method for mass production of fungal biopesticides since it provides micropropagules with higher conidia content. Rice grains for multiplication of *Trichoderma viride* has been reported by Kishore et al. (2014). The process described here utilized solid state fermentation technology using rice grain powder for mass production of *Trichoderma* inoculum. This procedure is very simple and could be used successfully in small and large scale production programmes. This method enables the manufacturer to preserve the inoculum for considerable period of time without loss in viability or quality. *Trichoderma* production generally requires 10-12 days or more which includes the time for inoculum production, formulation and curing/drying. By preserving the inoculum in store, the producer can immediately formulate the product when there is a requirement. In this process, drying process can be avoided since the formulation contains only 8% moisture which is the maximum moisture content for dry formulation of fungi as per the CIB guidelines. Thus, the time lag between the demand and supply of the product can be reduced to minimum by this procedure.

In addition, the *Trichoderma* spore mass could also be used directly for seed treatment, seed biopriming, soil/root treatment, wound dressing etc. Furthermore, the production of talc formulation of *Trichoderma* using rice powder is cost effective (B: C ratio – 3.2:1) when compared to the submerged inoculum in potato dextrose broth (B: C ratio – 2.6:1).

From the present study, it was concluded that sterilized rice powder supplemented with 3% dextrose formed an excellent base material for developing *Trichoderma* spore mass and could be a feasible method for inoculum production on large scale. Formulating talc with the sterilized rice powder+dextrose based inoculum @ 2% along with 8% sterile water sustained the viability of $6x10^8$ cfu g^{-1} at 180 days. This method can be evaluated for other biocontrol fungi also with necessary modifications.

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