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A Novel Method to Develop Paste Formulation of *Trichoderma harzianum*

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ABSTRACT

Keywords

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Biological control of diseases has been practiced as an alternative to control of plant disease using fungicides. *Trichoderma* spp. is the most widely exploited antagonistic fungi for the management of plant diseases world-wide. Though the biological control of plant diseases using *Trichoderma* spp. has been impressive and consistence under *in vitro* conditions, numbers of reports indicate wide variations in the disease control efficiency under field conditions. Formulation plays a significant role in determining the final efficacy of biocontrol agents. Several reports have emphasized the development of suitable formulations for the management of various plant diseases. The present study was proposed to develop a formulation of *T. harzianum* suitable for application through drip irrigation with longer shelf life. Among the 8 media tested, medium – M₃ has recorded the maximum mycelial dry weight of 654 mg. The highest population (11.6×10^{10} cfu/g) was recorded in the fresh formulation containing 10 per cent starch + copper sulphate 20 ppm at pH 4. Starch 10 per cent and copper sulphate 20 ppm adjusted to pH 4 has recorded significantly higher population even after 120 days of storage.

Introduction

The use of antagonistic micro-organisms for the control of various crop diseases has attracted considerable interest in recent years owing to the problems associated with large-scale use of synthetic chemical pesticides. Many researchers have demonstrated the potential of *Trichoderma* spp. in control of damping-off diseases of crop plants caused by *Pythium* sp. Species of genus *Trichoderma* are well documented fungal biological control agent (Chet *et al.*, 1981; Kleifield and Chet, 1992). *Trichoderma* based biocontrol agents possess better ability to promote plant growth and soil remediation activity compared to their counter parts. *Trichoderma* spp. have

gained wide acceptance as effective biocontrol agents against several plant pathogens. The use of antagonistic micro-organisms for the control of various crop diseases has attracted considerable interest in recent years owing to the problems associated with large-scale use of synthetic chemical pesticides. Many researchers have demonstrated the potential of *Trichoderma* spp. in control of damping-off diseases of crop plants caused by *Pythium* sp. (Le *et al.*, 2003), *Macrophomina phaseolina* (Khan and Gupta 1998) and *Rhizoctonia solani* (Lewis *et al.*, 1998). *T. viride*, *T. harzianum* and *T. virens* are being successfully used for the

control of some of the dreaded diseases like damping off, collar rots and wilts of horticultural crops.

Development of feasible and efficient delivery systems for the application of appropriate micro-organisms to the ecosystem is an important component of biocontrol technology (Lewis *et al.*, 1998). The formulation of microbial biomass represents a critical step advance in the commercial development of prospective biological control products. Several commercial formulations of *Trichoderma* are available in the market. The common formulations of *Trichoderma* spp. include dusts, pyrex/biomass, alginate pellets kaolin/starch/cellulose granules and extruded granules (Hebbal 1997; Lewis *et al.*, 1998). *Trichoderma* spp. is being extensively marketed as talc-based powder formulation (Nakkeeran *et al.*, 2002). The problems associated with talc-based powder formulations include dehydration and poor shelf life and loss in viability of propagules beyond three months of storage (Mukhopadhyay, 1994), which affects efficiency and marketability of the formulation (Singh *et al.*, 2000). Although many formulations for soil incorporation are in the market, not enough work has been carried out to develop a formulation available for application through drip irrigation. Effective control of soil-borne pathogens by biocontrol agents has been widely demonstrated (Singh, 2010). *Trichoderma* formulations as conidia or chlamyospore have restricted gradients of dispersion and reproduction in soils. The germination of conidia or chlamyospore is vulnerable to soil fungistasis (Xu *et al.*, 2004; Pan *et al.*, 2006). The introduced *Trichoderma* spores can lyse before germination, and growth of germinating spores cannot occur due to non-availability of continuous nutrient supply. This greatly limits the use of *Trichoderma* spores in controlling soil-borne diseases

(Papavizas, 1985; Hoitink and Boehm, 1999). Food supplements added to soil can maintain *Trichoderma* in a viable state and achieve a better biocontrol efficacy. It has been demonstrated that the development of liquid formulations of bio control agents in general has several advantages including high colony forming units, longer shelf life, zero contamination, ability to withstand environmental stresses and increased field efficiency over the conventional talc based formulation. Moreover when the talc based formulations are applied for the management of diseases of horticultural and plantation crops through micro irrigation techniques, blockage of nozzles often makes application laborious and cumbersome. Keeping these things in mind, the present study was proposed to develop a formulation of *T.harzianum* suitable for application through seed treatment and drip irrigation with longer shelf life.

Materials and Methods

Collection of *T.harzianum*

All the laboratory experiments were conducted at the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. The culture of *T. harzianum* was obtained from the National Bureau of Agriculturally important Insects (ICAR), Hebbal, and Bengaluru. The *T. harzianum* was further sub cultured on Potato Dextrose Agar (PDA). The cultures were maintained under refrigerated condition (4°C) and used periodically for preparing different formulations.

Standardization of suitable liquid medium for the growth of *T. harzianum*

Effect of eight different amendments of molasses based broth (Semi defined balanced medium) on the mycelial dry weight of

T. harzianum was studied and the composition of eight different media were used. The ingredients of urea, potassium monophosphate, magnesium sulfate, sodium citrate, sucrose were used for development of *T. harzianum* growth media (Table 1). From each medium 100 ml was transferred to 250 ml glass jar and sterilized at 121°C. The jars were transferred to laminar air flow chamber after cooling. Mycelial discs (5mm) from actively growing tips of 7 days old *T. harzianum* cultures were aseptically transferred to glass jars. The glass jars were loosely sealed with lids and incubated at laboratory condition (28±2°C) for seven days. After 7 days, the mycelial mats were collected and placed on funnel covered with Whatman No. 40 filter paper to drain out filtrates and the mycelial mats were oven dried at 85±2°C for 16 hours. The mats were weighed in electronic balance and recorded.

Development of paste formulations

In order to find out suitable paste formulation of *T. harzianum* which can sustain viability of conidia and chlamydo spores for longer period, the present experiment was conducted using three levels of food base in the form starch viz., 5.0, 7.5 and 10 per cent and 3 levels of copper sulphate viz., 20, 40 and 60 ppm and two levels of pH viz., 3.0, and 4.0 were tested for their effect on prolonging the viability of *T. harzianum* propagules.

Biomass of *T. harzianum* obtained from 7 days old culture grown on semi defined balanced medium (SDBM) medium No - 3 was concentrated 3 - 4 fold after harvest by filtration through a sieve with a pore size of No.100µm and mixed thoroughly in different combination of starch, copper sulphate and pH and stored in 250 ml glass jar. The amount of dry substance in the concentrate was approximately at 5 per cent (w/w). The viability of *T. harzianum* propagules in the

formulations were tested at monthly interval up to 120 days and expressed as colony forming unit/gm of formulations.

Analyzing the survival of *T.harzianum* propagules in the developed formulation

One g of formulation was taken and dissolved in 10 ml of sterile water and subsequent dilutions were made up to 10¹⁰. One ml from 10¹⁰ dilution was plated on sterile petriplates containing PDA and incubated for 48 hrs under laboratory conditions. The colonies were counted in each treatment and recorded as cfu/g of formulation.

Results and Discussion

Effect of molasses based media on the mycelial dry weight of *T. harzianum*

Effect of different amendments in molasses based media on the mycelial dry weight of *T. harzianum* was studied and the results are presented in Table 2. Among the 8 media tested, medium – M₃ has recorded the maximum mycelial dry weight of 654 mg followed by medium - M₁ with 631 mg. However medium M₃ and M₁ were statistically on par with each other. Medium M₅ has recorded the lowest mycelial dry weight of 50 mg.

Effect of paste formulation amended with copper sulphate at 20 ppm

The effect of different concentration of starch viz., 5, 7.5 and 10 per cent on the viability of *T. harzianum* was studied. Two levels of pH i.e. 3 and 4 were also studied for their effect on the viability of *T. harzianum* and the results are furnished in Table 3. All the six treatments have recorded higher colony forming unit immediately after formulation. However, the population started to decline after 30 days of storage under laboratory

conditions. The highest population of 11.6×10^{10} cfu/g was recorded in the fresh formulation containing 10 per cent starch + copper sulphate 20 ppm at pH 4. The lowest population of 9.0×10^{10} cfu/ g was recorded in the fresh formulation containing starch 7.5 per cent + copper sulphate at 20 ppm at pH 4 as well as pH 3.

After 90 days of storage at laboratory conditions paste formulation adjusted to pH 3 recorded zero population. However starch 10 per cent and copper sulphate 20 ppm adjusted to pH 4 has recorded significantly higher population (9.3×10^{10} cfu/ g) 90 days after preparation. The same formulation has retained significantly higher population (6.6×10^{10} cfu/ g) even after 120 days of storage. All other combinations recorded zero population 120 days after storage.

Effect of paste formulation amended in with copper sulphate at 40 ppm

The highest population of *T. harzianum* (10×10^{10} cfu/ g) was recorded in freshly prepared formulation containing starch 7.5 per cent and copper sulphate at 40 ppm at pH 4 and formulation containing starch 10 per cent + copper sulphate 40 ppm at pH 3. The fresh product containing at pH 3 has recorded the lowest population of *T. harzianum* (6×10^{10} cfu/ g). In all the treatments the population has started to decline after words.

Three combinations of paste formulation at pH 3 were not able to sustain the viability *T. harzianum* after 60 days of storage. However starch 10 per cent and copper sulphate 40 ppm combination at pH 4 sustained a population of 4.66×10^{10} cfu/g after 90 days of storage.

After 120 days of storage also the same treatment alone maintained a population of 1.3×10^{10} cfu/ g. All other treatments last the viability after 120 days of storage (Table 4).

Effect of paste formulation amended in with copper sulphate at 60 ppm

The concentration of copper sulphate was maintained at 60 ppm, the population of *T. harzianum* in the fresh product ranged from 2×10^{10} cfu/ g (starch 7.5% + pH at 3 + copper sulphate at 60 ppm) to 10.33×10^{10} cfu/ g (starch at 10% + pH at 4 + copper sulphate at 60 ppm). Three combination of paste formulation adjusted to pH 3 recorded zero population of *T. harzianum* after 60 days of storage. All the treatments have failed to maintain the viability of *T. harzianum* propagules after 120 days of storage (Table 5).

Biological control of diseases has been practiced as an alternative to control of plant disease using fungicides. *Trichoderma* spp. is the most widely exploited antagonistic fungi for the management of plant diseases worldwide. Though biological control of plant diseases using *Trichoderma* spp. has been impressive and consistence under *in vitro* conditions, number of reports indicate wide variations in the disease control efficiency under field conditions, Although external factors such as temperature, rainfall and nature of plant leaf surface may influence the efficiency of *Trichoderma* spp., nature of formulations and method of delivery are the two critical factors that influence the performance of *Trichoderma* spp. *in vivo*. Formulation plays a significant role in determining the final efficacy of biocontrol agents. Several reports have emphasized the importance of development of suitable formulations for the management of various plant diseases (Fravel and Larkins, 1996; Manikandan *et al.*, 2010). Normally biocontrol agents formulated using inert carriers have shorter shelf life than those formulated using food additives. Talc based formulations of *Trichoderma* spp. is being used widely for the management of plant diseases at present.

Table.1 Composition of media used for the production of *T. harzianum* in liquid culture

Media 1 (M₁) Molasses-30g Urea-0.5g Potassium monophosphate-2.0g Magnesium sulfate-0.2g Sodium citrate-0.3g Dist. Water – 1 litre	Media 2 (M₂) Molasses-30g Potassium monophosphate-2.0g Magnesium sulfate-0.2g Sodium citrate-0.3g Dist. Water – 1 litre	Media 3 (M₃) Molasses-30g Urea-0.5g Potassium monophosphate-1.0g Magnesium sulfate-0.2g Sodium citrate-0.3g Dist. Water – 1 litre	Media 4 (M₄) Molasses-30g Potassium monophosphate-2.0g Magnesium sulfate-0.2g Dist. Water – 1 litre
Media 5 (M₅) Molasses-30g Potassium monophosphate-2.0g Magnesium sulfate-0.2g Calcium carbonate-0.1g Dist. Water – 1 litre	Media 6 (M₆) Molasses-30g Urea-0.5g Potassium monophosphate-2.0g Magnesium sulfate-0.2g Calcium carbonate-0.1g Dist. Water – 1 litre	Media 7 (M₇) Molasses-30g Potassium monophosphate-2.0g Magnesium sulfate-0.2g Sodium citrate-0.3g Calcium carbonate-0.1g Ammonium sulfate-1.1g Sucrose-10g Dist. Water – 1 litre	Media 8 (M₈) Molasses-30g Potassium monophosphate-2.0g Magnesium sulfate-0.2g Ammonium sulfate-1.1g Sucrose-10g Dist. Water – 1 litre

Table.2 Effect of different amendments in molasses based media on the mycelial dry weight of *T. harzianum*

S.No.	Media	Mycelial dry weight of <i>T. harzianum</i> (mg)
1.	M ₁	631.000 ^a
2.	M ₂	109.333 ^c
3.	M ₃	654.000 ^a
4.	M ₄	124.000 ^c
5.	M ₅	50.000 ^c
6.	M ₆	348.333 ^b
7.	M ₇	191.667 ^{bc}
8.	M ₈	217.333 ^{bc}

Values are means of 3 replications. In a column, means followed by common letters are not significantly different at the 5% level by DMRT.

Table.3 Effect of paste formulation of *T. harzianum* amended with copper sulphate (CuSO₄) at 20ppm

S.No.	Treatments	Population of <i>T. harzianum</i> in 10 ¹⁰ cfu/g				
		0 th day	30 th day	60 th day	90 th day	120 th day
1.	T ₁ – (Starch 5% +pH 4 + CuSO ₄ 20ppm)	10.00	3.00	1.66	0.33	0.00
2.	T ₂ – (Starch 7.5% + pH 4 + CuSO ₄ 20ppm)	9.00	8.60	5.30	3.00	0.66
3.	T ₃ – (Starch 10% + pH 4 + CuSO ₄ 20ppm)	11.60	11.30	10.00	9.30	6.66
4.	T ₄ – (Starch 5% + pH 3 + CuSO ₄ 20ppm)	10.00	9.60	00.00	0.00	0.00
5.	T ₅ – (Starch 7.5% + pH 3 + CuSO ₄ 20ppm)	9.00	10.30	00.00	0.00	0.00
6.	T ₆ – (Starch 10% + pH 3 + CuSO ₄ 20ppm)	9.60	11.00	3.30	0.00	0.00

Values are means of 3 replications.

Table.4 Effect of paste formulation of *T. harzianum* amended with copper sulphate (CuSO₄) at 40ppm

S.No.	Treatments	Population of <i>T. harzianum</i> in 10 ¹⁰ cfu/g				
		0 th day	30 th day	60 th day	90 th day	120 th day
1.	T ₁ – (Starch 5% + pH 4 + CuSO ₄ 40ppm)	8.00	4.00	0.60	0.00	0.00
2.	T ₂ – (Starch 7.5% + pH 4 + CuSO ₄ 40ppm)	10.00	4.33	3.33	1.00	0.00
3.	T ₃ – (Starch 10% + pH 4 + CuSO ₄ 40ppm)	9.30	5.66	5.66	4.66	1.30
4.	T ₄ – (Starch 5% + pH 3 + CuSO ₄ 40ppm)	9.00	4.66	0.00	0.00	0.00
5.	T ₅ – (Starch 7.5% + pH 3 + CuSO ₄ 40ppm)	6.00	5.33	0.00	0.00	0.00
6.	T ₆ – (Starch 10% + pH 3 + CuSO ₄ 40ppm)	10.00	5.66	0.00	0.00	0.00

Values are means of 3 replications.

Table.5 Effect of paste formulation of *T. harzianum* amended with copper sulphate (CuSO₄) at 60ppm

S. No.	Treatments	Population of <i>T. harzianum</i> in 10 ¹⁰ cfu/g				
		0 th day	30 th day	60 th day	90 th day	120 th day
1.	T ₁ – (Starch 5% + pH 4 + CuSO ₄ 60ppm)	9.60	6.00	1.00	0.00	0.00
2.	T ₂ – (Starch 7.5% + pH 4 + CuSO ₄ 60ppm)	9.66	3.33	0.00	0.00	0.00
3.	T ₃ – (Starch 10% + pH 4 + CuSO ₄ 60ppm)	10.33	4.00	2.66	1.00	0.00
4.	T ₄ – (Starch 5% + pH 3 + CuSO ₄ 60ppm)	8.33	2.66	0.00	0.00	0.00
5.	T ₅ – (Starch 7.5% + pH 3 + CuSO ₄ 60ppm)	2.00	2.00	0.00	0.00	0.00
6.	T ₆ – (Starch 10% + pH 3 + CuSO ₄ 60ppm)	9.00	3.33	0.00	0.00	0.00

Values are means of 5 replications.

However, blocking of nozzles when applied through sprayers and drip systems, bulkiness and allergy related problems warrant development of novel formulations of *Trichoderma* spp. to meet today's changing agricultural scenario.

Among the 8 molasses based media tested for supporting the mycelial growth of *T. harzianum*, M₃ medium was found to be superior followed by M₁ medium. However wide variations were observed among the 8 media tested. In addition to molasses as a carbon source, addition of other ingredients like urea (0.5g), potassium monophosphate (1–2 g) and traces of magnesium sulphate and sodium citrate were found to be essential for mycelial growth of *T. harzianum* *in vitro*. Mass multiplying biocontrol agents using optimum growth conditions is very important, since it plays a vital role in sustaining the viability in formulated product. Substances required to sustain culture viability during storage can be added not only to harvested biomass but also to the fermentation broth at the outset of inoculum production (Kolombet *et al.*, 2008). M₃ – medium which best supported the mycelial growth of *T. harzianum* was selected and different paste formulations were prepared from the biomass cultivated using the above medium. In the present study, using this formulation, initial population of 11.60×10^{10} cfu / g was obtained which gradually decreased to 11.3, 10, 9.3 and 6.7×10^{10} cfu /g after 30, 60, 90 and 120 days of storage under laboratory condition. In other combinations though initial population was comparable with earlier combination, significant reduction in population was observed after 60 days of storage. Among two levels pH tested, pH 4 was found to be optimum in reducing the metabolic activity of *T. harzianum* while retaining the viability of propagules. At pH 3, most of the propagules of *T. harzianum* lost the viability after 60 days of storage under laboratory conditions. This

may be due to the fact that *Trichoderma* may produce different types of spores, such as chlamyospores and conidia at different pH levels. Usually the mycelial form will not grow and tolerate acidification process resulting in proliferation of spores in the formulation. The finding is in agreement with the results of Kolombet *et al.*, (2008). They reported that *T. asperellum* retained the viability for more than 4 months when stored at 30°C at pH 4. However, the viability was lost when the pH of paste formulation of *T. asperellum* was maintained at pH 3. Copper ions operate as an enzymatic inhibitor, primarily blocking cytochrome activity (Russel *et al.*, 1997). Copper sulphate at more than 0.01 percent concentration can have a fungicidal effect. However in small amounts a respiratory shock occurs in cell resulting in the slowdown of cell development, but not in the loss of cell viability. We have observed more number of chlamyospores in paste formulation containing 20 ppm of copper sulphate than in formulations containing 40 and 60 ppm of copper sulphate. When copper sulphate was added in the formulation of *T. asperellum*, the mycelium completely lysed while chlamyospore and conidia had been produced. The paste formulation containing starch 10 per cent + copper sulphate at 20 ppm with pH of 3 supported the production of more chlamyospore meanwhile 60 ppm copper sulphate supported proliferation of conidia (Kolombet *et al.*, 2008).

Shelf life of the formulated product of biocontrol agents plays a significant role in successful marketing. In general, antagonists multiplied in an organic food base having longer shelf life than the inert or inorganic food bases. Shelf life of *Trichoderma* in coffee husk was more than 18 months. Talc, peat, lignite and kaolin based formulation of *Trichoderma* have a shelf life of 3-4 months. The viable propagules of *Trichoderma* in the talc formulation were reduced by 50 per cent

after 120 days of storage (Sankar and Jayarajan, 1996).

In the present study, we prepared a paste formulation of *T. harzianum* mainly constituted of starch and copper sulphate. This formulation has a positive effect on the viability and persistence of conidia up to 4 month, this biological control approach is interesting from an environmental perspective, but also for the small scale production, with the added advantage that our paste formulation may bring down the cost of production to be commercially viable.

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