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ORIGINAL ARTICLE

The efficacy of *Trichoderma asperellum* and *Trichoderma virens* inocula for the control of *Fusarium solani* the causative agent of damping off disease of *Allium cepa* L. under green house conditions

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ABSTRACT
Trichoderma spp. isolated from the soils of onion fields in Sri Lanka were tested for their effect on the control of Fusarium solani, the causative agent of damping off disease in Allium cepa L. Innocula of the more effective Trichoderma isolates i. e. Trichoderma asperellum, Trichoderma virens were prepared for green house tests. Rice bran + saw dust (Medium I), molasses + yeast (Medium II) were evaluated for the mass production of Trichoderma virens and Trichoderma asperellum. Both T. virens and T. asperellum showed a significantly ($p \leq 0.05$) high level of spore production in molasses + yeast medium 14 days after inoculation producing $1.15 \pm 0.05 \times 10^{10}$ and $1.36 \pm 0.03 \times 10^{10}$ spores/ mL (n=3) respectively as compared to $1.92 \pm 0.07 \times 10^{8}$ and $2.23 \pm 0.23 \times 10^{8}$ spores/mL (n=3) in the rice bran + saw dust medium indicating that molasses + yeast medium is more suitable for mass production of Trichoderma spp. Medium consisting of Talc at a rate of 1: 2 (v/w) proved to be suited as a carrier medium. The antagonistic potential of the prepared inocula were tested for their effect on the control of damping off pathogen. Inocula of Trichoderma asperellum and Trichoderma asperellum and Trichoderma virens in combination were introduced as soil applications or as seed priming treatments. Both methods reduced disease incidence of damping off disease significantly ($p \leq 0.05$) indicating that the Trichoderma inocula prepared can be used effectively against damping off disease of Allium cepa L.
KEYWORDS: *Allium cepa L., damping off disease, formulation, Fusarium solani, seedlings, Trichoderma virens, Trichoderma asperellum*

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INTRODUCTION

Allium cepa L. (Big onion) is used as a condiment in many countries of the world and is an important cash crop grown in Sri Lanka. Damping off disease of *Allium cepa* L. seen at the nursery stage is one of the most important diseases caused by soil-borne fungal spp. *Fusarium, Pythium* and *Rhizoctonia* either singly or in combination resulting in damping off disease. This disease causes considerable production constraints in the onion fields [13]. This disease may manifest before or after emergence of seedlings *i.e.* pre-emergence or post emergence damping off resulting in severe seedling mortality.

Planting seeds treated with fungicides, Thiram, Homai, soil treatment with fungicides, Brassicol, Benlate, Captan, Cresent and soil solarisation are being carried out to control damping off disease in Sri Lanka [4]. However, due to the disadvantages associated with fungicide applications such as development of

resistance by the pathogen when exposed continuously to the chemical, high cost, deleterious effects on soil organisms and most importantly the adverse effects on the environment and associated health hazards, alternative disease management strategies have to be developed [15].

Biological control of plant pathogens is devoid of most of the above limitations whilst being safe and sustainable. Species of *Trichoderma* have been identified and evaluated as highly effective biological control agents against many soil-borne phytopathogenic fungi including *Fusarium* spp. and *Pythium* spp.[3, 2, 8].

In the present study, the control of damping off disease of seedlings of *Allium cepa* L. caused by *Fusarium solani* was evaluated under green house conditions using *Trichoderma asperellum* and *Trichoderma virens* isolated from local onion fields.

The objectives of this study were to prepare an inoculum by culturing, two *Trichoderma* spp. *i.e. Trichoderma asperellum and Trichoderma virens* shown to be effective in controlling the growth of *Fusarium solani* under laboratory conditions using a suitable low cost mass production medium and to test the efficacy of inoculum for effective control of damping off disease of *Allium cepa* L. seedlings under green house conditions.

MATERIAL AND METHODS

Collection of seedling and soil samples from the field

Diseased big onion seedlings at different stages of growth (7 days-30 days after cultivation) and soil samples each of 5 g from the top 5-10 cm depth were collected randomly from fifty-five onion fields in the Matale and Anuradhapura districts during the *yala* season. The locations of the sites were recorded through GPS receiver (Garmin) and a map of sites was constructed using ArcMap Version 9.2 (Figure 1).

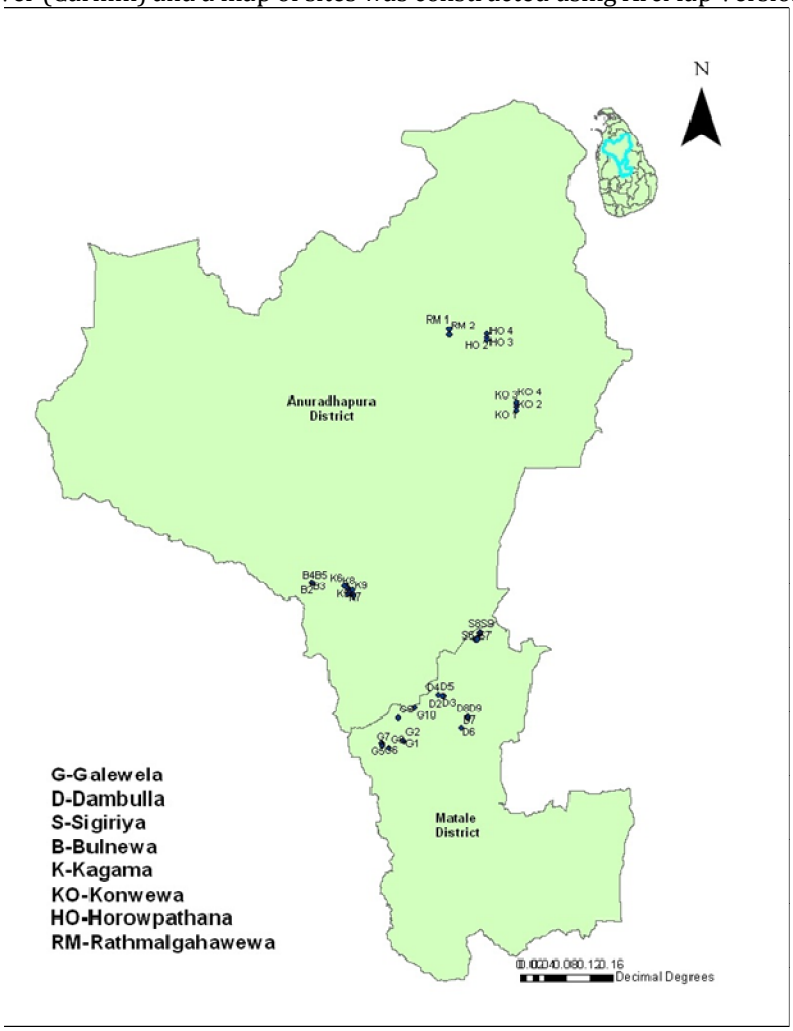

Figure 1: Sampling fields

The fungal species present in the diseased seedlings were also isolated and identified. Similar fungal genera *i.e. Aspergillus, Curvularia, Fusarium, Penicillium, Alternaria, Mucor, Sclerotium* were associated

with the seedling samples collected from both Matale and Anuradhapura districts. *Fusarium solani* was isolated from the diseased samples collected from onion fields and was confirmed to cause damping off disease [6].

Two *Trichoderma* spp. *i.e. Trichoderma asperellum* and *Trichoderma virens* were selected as they had the highest antagonistic activity against *Fusarium solani*, highest growth rate and highest sporulation capacity [7].

The NCBI GeneBank accession numbers obtained for *Trichoderma asperellum, Trichoderma virens* and *Fusarium solani* used in this study are MG198706, MG199587 and MF685335 respectively.

Selection of a low cost culture medium for the culture of the *Trichoderma* spp.

Two low cost media (designated as I and II) were tested for culture of *Trichoderma virens* and *Trichoderma asperellum*. The two *Trichoderma* spp. were cultured on PDA at room temperature for 2 weeks until well sporulated and added to the two media separately as described below:

Medium I

Composition:

Saw dust- 1 kg, Rice bran- 100g, Soy flour-10g, $CaCO_3$ - 20g, $MgSO_4$ – 2g, Glucose – 20g and water 1 L (16). One hundred gram each of the medium was added to 125 mL plastic jars and autoclaved at 121 ^oC for 20 minutes under 15 Psi. Conidial suspensions were prepared from one week old cultures of each *Trichoderma* sp. and the concentration was adjusted to $1x10^8$ conidia/mL water. Three mL of conidial suspension of each *Trichoderma* sp. was added separately to the culture medium I in each plastic jar under aseptic conditions and incubated in room temperature. One gram samples were drawn from the jars 14 days after inoculation and a dilution series was prepared using sterilized distilled water. Spore concentration of each sample was estimated under the microscope using a haemocytometer.

Medium II

Composition:

Molasses 30 g, 5 g yeast extract in 1 L of distilled water.

1 cm diameter discs of the culture were cut with a cork borer and eight agar discs were inoculated into each flask containing 500 mL molasses yeast broth and shaken in 180 rpm using a rotary shaker. Growth of *Trichoderma asperellum* and *Trichoderma virens* in the medium was monitored using a haemocytometer for 14 days.

Incorporation into carrier medium

The entire contents of *Trichoderma asperellum* grown in 500 mL molasses yeast extract medium for 12 days was incorporated into the sterile carrier medium *viz*. talc at a rate 1:2 (v/w) ratio and maintained under aseptic conditions. The contents were thoroughly mixed, shade dried for 6 days and stored in polythene bags at room temperature. A sample of 0.05 g of the product was withdrawn from the bag before storage and 21 days after storage and evaluated for the presence of viable *Trichoderma asperellum* colonies using the Warcup method [19].

Effect of storage period on the viability of the *Trichoderma asperellum* formulation (formulation I)

The effect of storage period on the viability of the *Trichoderma asperellum* formulation was tested. The powder formulation of *Trichoderma asperellum* (initial inoculum of 1×10^7 CFU/g) was stored in sealed polythene bags at room temperature (30^oC). After 1, 2 and 3 months of storage, 1 g of the formulation was used for serial dilution tests. *Trichoderma asperellum* colonies were enumerated on the Potato Dextrose Agar +Tetracycline plates after 7 days of incubation at 30^oC and the size of populations were expressed as colony forming units (CFU) per g of the powder formulation.

Compatibility studies among *Trichoderma asperellum* and *Trichoderma virens*

To test the compatibility of *Trichoderma asperellum* with *Trichoderma virens* according to dual-culture technique, an *in vitro* bioassay was done on PDA medium. One centimeter diameter discs from the growing edges of pure cultures of both *Trichoderma* spp. (*Trichoderma asperellum* and *Trichoderma virens*) were co-inoculated onto PDA plates and growth pattern was observed.

Preparation of formulation containing both *Trichoderma asperellum* and *Trichoderma virens* (formulation II)

Four discs each of 1 cm diameter were cut from each *Trichoderma* spp. and inoculated together into 500 mL molasses yeast broth and shaken at 180 rpm using a rotary shaker for 12 days. Both *Trichoderma* spp. grown together in molasses yeast extract medium was incorporated into the sterile carrier medium *viz.* talc at a 1:2 (v/w) ratio under aseptic conditions. The contents were thoroughly mixed; shade dried for 6 days and stored in polythene bags at room temperature ($30 \pm 2\ ^o C$). Initial and 2 months after storage of formulation *Trichoderma asperellum* and *T. virens* populations were determined using dilution plate technique.

Assessment of moisture level of the formulations

Initial moisture levels of the two formulations were analyzed by taking 50 g of each formulation (formulation I and II) into crucibles and heating at 100 ^{o}C until a constant weight was achieved.

Test on antagonistic activity of *Trichoderma asperellum* and *Trichoderma virens* under greenhouse conditions

Eight centimeter diameter plastic pots were filled with 100 g of the potting mixture consisting of partial sterilized compost with aerated steam (100^{o}C for 1h) and sieved, autoclaved soil with saturated steam under 15 psi pressure (121^{o}C for 30 min.). 0.097 g of *A. cepa* L. seeds were planted in each pot. Eight discs of 1 cm diameter *Fusarium solani* maintained on PDA for seven days was inoculated to 500 mL of Molasses yeast extract medium and allowed to grow for 12 days. The entire biomass along with medium was then incorporated into sterile Medium - saw dust, rice bran, soy flour, lime, $MgSO_4$, Glucose medium under aseptic conditions. This *Fusarium solani* inoculum was inoculated to the potting mixture at a concentration of 5.0 X 10^3 CFU/g. *Trichoderma asperellum* was inoculated to the potting mixture at a concentration of 1.2 X 10^7 CFU/g. *Trichoderma* spp. *i.e. Trichoderma virens* and *Trichoderma asperellum* were inoculated to the potting mixture at a concentration of 0.6 X 10^7 CFU/g and 0.6 X 10^7CFU/g respectively. Complete randomized design was used with five replicates per treatment and the experiment was repeated three times. The scheme of treatments used is shown in Table 1.

Table 1 Treatments tested under green house conditions

Abbreviation	Treatment
T 1	*Trichoderma virens* and *Trichoderma asperellum* combined preparation in talc (soil inoculation) with *Fusarium solani* soil inoculation
T 2	*Trichoderma asperellum* in talc (soil inoculation) with *Fusarium solani* soil inoculation
T 3	*Fusarium solani* soil inoculation (Control)
T 4	*Fusarium solani* soil inoculation with 1000 µL (1 g/L) Captan
T 5	*Trichoderma virens* and *Trichoderma asperellum* together in talc applied as seed treatment with *Fusarium solani* soil inoculation
T 6	*Trichoderma asperellum* in talc applied as seed treatment with *Fusarium solani* soil inoculation

The effect on *A. cepa* L. seedling damping off disease incidence was assessed by visual observations after 20 days. Mean disease incidence in seedlings was calculated after 20 days. The means of five replicates were analyzed using ANOVA and Tukey's test at 5 % significant level with Minitab 16 statistical analysis software. Percentage data were transformed into arcsine values.

Disease incidence was calculated as number of infested seedlings showing any of the damping off symptoms (i.e. yellowing and wilting of leaves, stunting of seedlings - seedling length ≤ 12.0 cm, collapsed seedlings and/or completely dead seedlings) out of total number of *A.cepa* L. seedlings observed

$$\text{Disease incidence} = \frac{\text{Number of infected seedlings}}{\text{Total number of seedlings assessed}} \times 100$$

RESULTS

Selection of a low cost mass culture medium for the *Trichoderma* spp.

Trichoderma asperellum and *Trichoderma virens* showed a significantly ($P \leq 0.05$) high level of spore production in molasses + yeast medium 14 days after inoculation producing $1.15 \pm 0.05 \times 10^{10}$ and $1.36 \pm 0.03 \times 10^{10}$ spores/ ml (n=3) for *T. virens* and *T. asperellum* respectively as compared to $1.92 \pm 0.07 \times 10^8$ and $2.23 \pm 0.23 \times 10^8$ spores/ml (n=3) in the rice bran + saw dust medium.

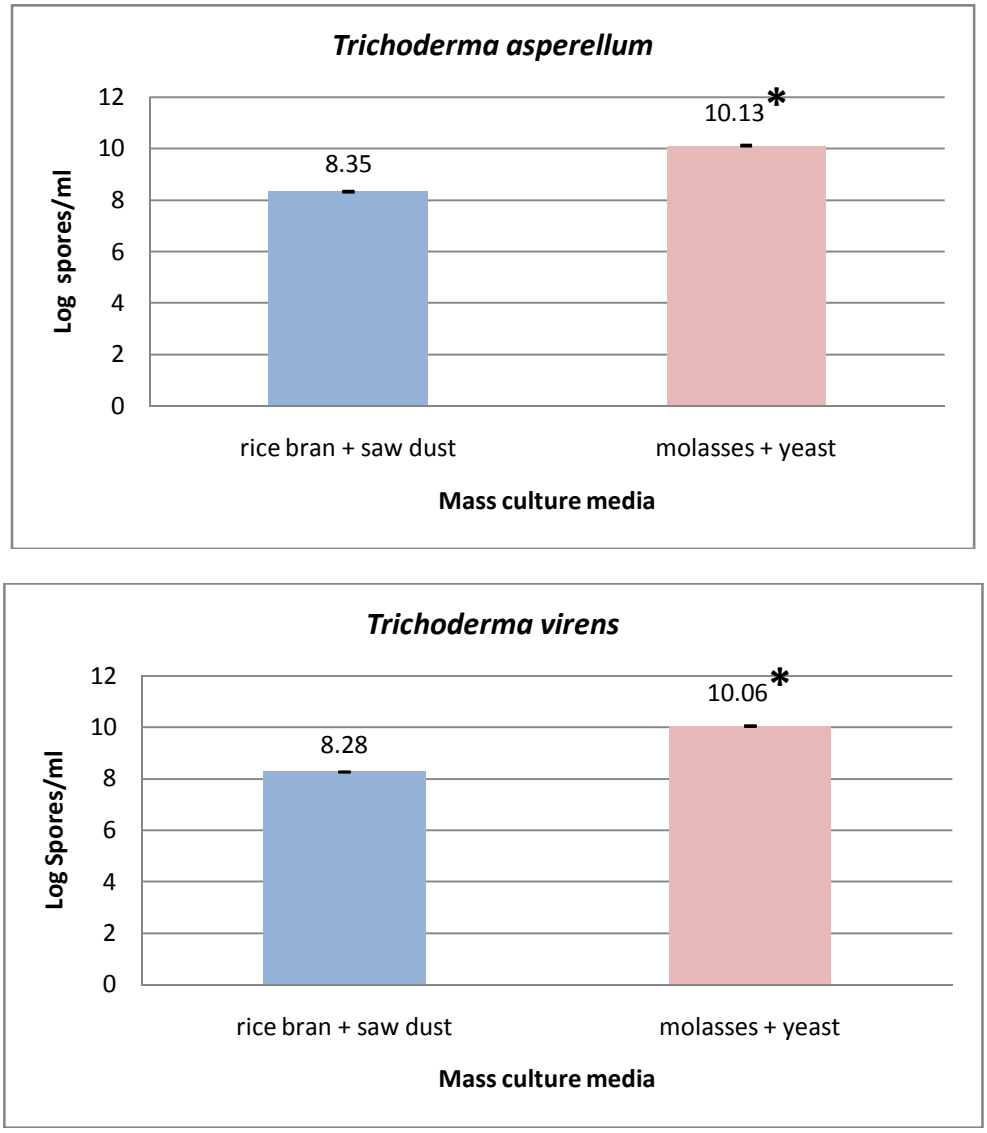

Figure 2: Comparison of spore concentrations of *Trichoderma* spp. in the rice bran + saw dust (Medium I) and molasses + yeast (Medium II) mass multiplication media

Each bar represents the mean & the standard error values from three independent experiments. The asterisk (*) indicates mass culture medium with a significantly different spore production ($p \leq 0.05$) by t-test, which suggest that molasses + yeast medium was more suitable for mass production of the two *Trichoderma* spp.

Effect of storage period on the viability of the *Trichoderma asperellum* in formulation I

The number of colony forming units of *Trichoderma asperellum* in the carrier medium decreased gradually with storage time (Table 2).

Table 2 Effect of storage period on viability of *Trichoderma asperellum* in formulation I

Storage period (months)	0	1	2	3
cfu/g of *Trichoderma asperellum*	1×10^{7}	3×10^{6}	2×10^{5}	4×10^{4}

Compatibility of *Trichoderma asperellum* and *Trichoderma virens*

Results of the compatibility test showed that *Trichoderma asperellum* and *Trichoderma virens* were compatible in dual cultures showing no inhibition zones. Fifty percent growth of *Trichoderma asperellum* and 50 % growth of *T. virens* could be observed after co-culture on PDA medium.

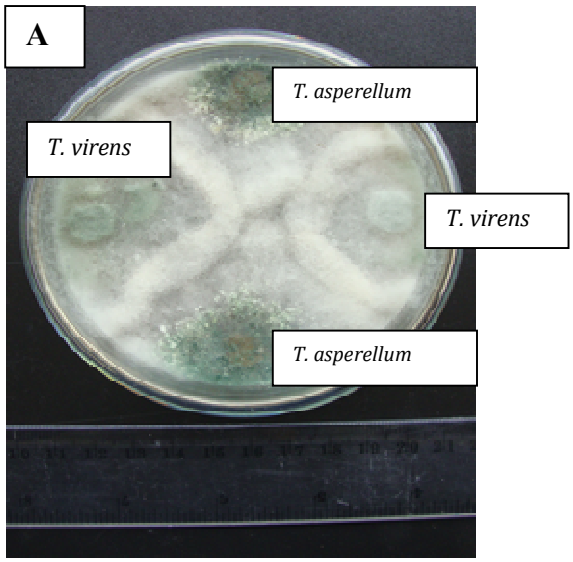

Figure 3: Co-cultivation of *Trichoderma virens* and *T. asperellum* (A) upper side of inoculated PDA plate; 50 % growth of *Trichoderma virens* and 50 % growth of *T. asperellum*

Survival of inoculum with *Trichoderma asperellum* and *Trichoderma virens* (formulation II)
Talc supported the survival of both *Trichoderma* spp. maintaining 1.5 $\times 10^5$ CFU/g for each *Trichoderma* spp. after two months.

Assessment of moisture level of the formulations (formulation I and II)
Moisture level of the formulation I and formulation II were 26.38 % and 31.40 % respectively.

Tests on antagonistic effect of *Trichoderma asperellum* and *Trichoderma virens* under greenhouse conditions

Table 3 Effect of *Trichoderma* spp. on damping off incidence of *A.cepa* L. seedlings under green house conditions

Treatment**	**Disease Incidence %***
T 1	6.076 (14.192) ^{bc}
T 2	9.512 (17.842) ^{b}
T 3	59.000 (50.308) ^{a}
T 4	10.204 (18.544) ^{b}
T 5	2.288 (5.534) ^{c}
T 6	3.976 (10.282) ^{bc}

*Means of five replicates in each treatment. Figures in parentheses are arc sin transformed values. Values with different letters are significantly different ($p \leq 0.05$)

There was a significant difference ($p \leq 0.05$) in disease incidence in seedlings treated with *Trichoderma* spp. and fungicide (T1, T2, T4, T5 and T6) compared to *Fusarium solani* soil inoculated control pots (T 3). The lowest disease incidence (2.288 %) was observed with T 5 *i.e. Trichoderma virens* and *Trichoderma asperellum* applied together as a seed treatment. The % DI of all seedlings treated with *Trichoderma* spp. were significantly different ($p \leq 0.05$) from controls. DI of seedlings treated with the combined *Trichoderma* spp. inoculum (T 1) was not significantly different from those that were inoculated with a single *Trichoderma* sp. (T 2) *i.e.* as soil inoculation. Similarly, there was no significant different between seeds treated with the combined *Trichoderma* spp. inoculum (T 5) and those that inoculated with a single *Trichoderma* sp. (T 6). Accordingly both *Trichoderma* spp. (*Trichoderma asperellum* alone or *Trichoderma asperellum* and *Trichoderma virens* combination) either as seed coating or soil treatment and chemical control given by Captan are equally efficient for controlling damping off disease.

DISCUSSION

As damping off disease caused by *Fusarium solani* causes substantial losses to onion seedlings at the nursery stage, it is important that effective control measures are in place.

Two locally available , natural substrates *i.e.* a sawdust based medium and molasses yeast medium were evaluated as a suitable medium for culture and the molasses yeast medium was selected to be more suitable as it facilitated sufficient sporulation (10^{10} spores/mL) and growth of both *Trichoderma* isolates tested. High numbers of mature chlamydospores were also produced in the molasses yeast medium which may allow prolonged shelf life of the final preparation. As molasses is a byproduct of the sugar industry, it is accessible locally at a fairly low cost which makes molasses yeast a suitable medium in many aspects.

Molasses has been reported to be suitable of producing fungal inocula on a higher scale. [10] also used molasses yeast extract medium to multiply *T. viride* as an inoculum and [14] produced large batches of *Gliocladium virens, Trichoderma hamatum, Trichoderma harzianum, Trichoderma viride* and *Talaromyces flavus* utilizing liquid fermentation of molasses and brewer's yeast. [17] showed that vermiculate soil and wheat bran medium can be utilized as mass multiplication and carrier medium for *Trichoderma* spp. to control damping off and wilt diseases of *Phaseolus vulgaris* L.

Once a suitable low cost medium for the culture of the bio control agent is selected, a carrier medium for field applications as well as modes of application have to be selected.

(5) tested efficacy of eight different carrier materials and combinations to formulate a suitable *Trichoderma harzianum* based bio-fungicide for controlling foot and root rot diseases of brinjal caused by *Sclerotium rolfsii* and found that four combinations of carrier materials based *Trichoderma harzianum* bio-fungicides such as (1) wheat bran + rice bran, (2) wheat bran + mustard oil cake (MOC) + rice bran, [3] khesari bran + rice bran, and [4] khesari bran +MOC+ rice bran were suitable.

In the present study, Talc was selected as a suitable medium because it supported the survival of *Trichoderma* spp. up to three months.

Similarly, [10] screened different materials *i.e.* gypsum, peat, kaolin, lignite, talc, fly ash as carrier media for the *Trichoderma viride* multiplied in molasses yeast extract medium and found that talc and gypsum were significantly superior in maintaining the survival of *T. viride* even after 150 days of storage. Rajapakse *et al*.[16] utilized a saw dust based medium as both mass culture and carrier media for *Trichoderma harzianum* for management of corm rot of Kiriala (*Xanthosoma sagittifolium* (L) SCHOTT). [11] have tested two carrier materials *i.e.* Talc and gypsum and revealed that the shelf life period was high in talc based formulation. [9] utilized composted chicken manure as a medium for the production and delivery of *Trichoderma virens* for weed control. [12] also tested the effect of different temperatures on the shelf life of *Trichoderma* based talc and lignite formulations and concluded that these products could be safely stored at room temperature for up to 90 days.

Several methods have been recommended for application of bio-fungicides for the successful management of plant diseases by several workers. The most common application strategies are seed biopriming, seedling dip (suitable for the crops where transplanting is practiced), soil application and foliar spray [18]. [2] used coated seeds and soil treatment with different combinations of *Trichoderma* spp. to control *Fusarium* rot of lentil and found out both reduction of disease severity and growth enhancement.

Two methods *i.e.* seed coating and soil inoculation were tested for the introduction of the prepared *Trichoderma* inoculum under greenhouse conditions and the results showed that seed priming and soil treatment with *Trichoderma* spp. were suited to be used in the field trials.

However, inoculation of the two *Trichoderma* spp. in combination did not show an increased level of control of the disease when compared with their separate inoculations. Similar inoculations of more than one *Trichoderma* spp. are sometimes reported to be effective in controlling pathogens. By using a combination of *T. harzianum*, *T. asperellum* and *T. virens* it was possible to reduce disease incidence percentage of cucumber fields exposed to *Fusarium* pathogens such as *F. solani* and *F. oxysporum* as agents of root and stem rot cucumber under greenhouse conditions [1]. In the present study the level of control achieved by the *Trichoderma* inoculum was not significantly different from that achieved by the fungicide treatment.

CONCLUSION

The present evaluation thus gave clear indication that *Trichoderma virens* and *Trichoderma asperellum* isolated from soils of *A. cepa* L. growing areas can be effectively used in the management of *A. cepa* L. damping off disease caused by *Fusarium solani* under green house conditions.

DECLARATION OF INTERESTS

None.

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