Journal of Biofertilizers & Biopesticides
ISSN: 2155-6202

Navaneetha et al., J Biofertil Biopestici 2015, 6:1
DOI: 10.4172/2155-6202.1000149

Research Article Open Access

Liquid Formulation of *Trichoderma* Species for Management of Gray mold in Castor (*Ricinus communis* L.) and Alternariaster Leaf Blight in Sunflower (*Helianthus annuus* L.)

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Received date: September 17, 2014; **Accepted date**: December 22, 2014; **Published date**: December 29, 2014

Abstract

The composition, concentration, shelf life and inconsistent performance are the major concern in formulation of bioagents in general and *Trichoderma* spp. in particular. To overcome some of these problems, Suspension Concentrate (SC) formulation of a potential isolates of *Trichoderma* viz., *T. harzianum* Th4dSC and consortium of *T. harzianum* Th4d SC and another species *T .asperellum* Tv5 SC were developed using conidial biomass of these bioagents produced by a production process supporting a higher quantities of biomass and viable propagules with better shelf life and persistence on sprayed surfaces. In this study, twenty five selected strains of *Trichoderma* were screened in vivo against foliar diseases (Alternariaster Leaf Blight (ALB) of sunflower and Botryotinia Gray Mold (BGM) of castor). Among these strains, *T. harzianum* Th4d and *T. asperellum* Tv5 were able to produce mycolytic, defense enzymes and able to control two diseases effectively. Further a study was conducted to standardize the dose of SC formulation of *Trichoderma* for foliar application and seed treatment. The highest antagonistic activity was achieved with concentration of 2×10^7 conidia/ml. The two selected strains were able to reduce severity of BGM and ALB under greenhouse conditions. In field trials (rainy season of the years 2009-10 and 2010-11), seed treatment, foliar application of *T. harzianum* Th4d SC at 2 ml/l and consortium formulations (*T. harzianum* Th4dSC+ *T. asperellum* Tv5SC at 1 ml/l) effectively reduced disease severity (ALB reduction up to 50-55% and BGM up to 55-65%). In addition, these applications enhanced yield of sunflower and castor compared to untreated control. Application of *T. harzianum* Th4dSC at 2 ml/l and consortia *T. harzianum* Th4dSC + *T. asperellum* Tv5SC at 1 ml/l showed better persistence with log CFU (colony forming unit) 7.61 and 8.45 respectively on sprayed leaves of sunflower population levels of log CFU 10.73 and 10.71 on capsules of castor at 15 days after first spray. The liquid formulations of both the strains retained good shelf life with viable spore counts of log CFU of 8.0 to 7.4 at 540 days when stored at room temperature. Thus, the efforts resulted in identification and development of suspension concentrate formulation with long shelf life of two potential *Trichoderma* strains for the first time for foliar disease management.

Keywords: *Alternariaster helianthi*; *Botryotinia ricini*; Suspension concentrate formulation; Shelf life; *Trichoderma harzianum*; *Trichoderma asperellum*

Abbreviations

SC: Suspension Concentrate; ALB: Alternariaster Leaf Blight; BGM: Botryotinia Gray Mold; TSM: Trichoderma Specific Medium; PDA: Potato Dextrose Agar; OMA: Oat Meal Agar Medium; MSM: Molasses-Soy Medium; HDPE: High-Density Polyethylene; ITS: Inter Transcribed Spacers; BCA's: Biological Control Agents; FYM: Farm Yard Manure; ISR: Inducing Systemic Resistance; WDP: Wettable Dry Powder; PCR: Polymerase Chain Reaction; NCBI: National Center for Biotechnology Information; BLAST: Basic Local Alignment Search Tool; NAIMCC: National Agriculturally Important Microbial Culture Collection; RBD: Randomized Block Design; ANOVA: Analysis of Variance

Introduction

Trichoderma is considered as most efficient biocontrol agents and have attracted considerable scientific attention as they are considered as promising alternative to chemical fungicides against many plant pathogens. Major mechanisms involved in the biocontrol activity of *Trichoderma* spp. are competition for space and nutrients, production of diffusible and/ or volatile antibiotics and hydrolytic enzymes like chitinase and β-1,3- glucanase. These hydrolytic enzymes partially degrade the pathogen cell wall and leads to its parasitization [1]. Many recent findings suggest that plant development and biochemistry are strongly affected by *Trichoderma* strains. Specific strains of fungi in the genus *Trichoderma* colonize and penetrate plant root tissues and initiate a series of morphological and biochemical changes in the plant, considered to be part of the plant defense response, which in the end leads to Induced Systemic Resistance (ISR) in the entire plant. *Trichoderma* species have the ability to interact with roots of diverse plant species leading to Induced Systemic Resistance (ISR) responses to a wide spectrum of pathogens and adverse environmental conditions [2,3].

Other ecological success of this genus is its capability to synthesize antagonistic compounds (proteins, enzymes and antibiotics) and growth promoting substances (vitamins, hormones and minerals) enhance their bio-control activity [4].

Castor plant (*Ricinus communis* L.) is a non-edible oilseed crop with unique oil features for the chemistry industry. The crop was very important in United States in the mid and late nineteenth century and also during First World War. The crop lost its importance in developed countries, but in India and Brazil it has remained as the most important non-edible oilseed crop of the arid and semi-arid regions [5,6]. One of the most destructive diseases of castor is gray mold, caused by the fungus *Botryotinia ricini* (Godfrey) Whetzel. Actually, it is the anamorphic phase of *B. ricini*, known as *Amphobotrys ricini* (N.F. Buchw.) Hennebert, that is responsible for disease epidemics and heavy yield losses frequently observed in castor crops. The first epidemic outbreak caused by this fungus was reported by H.E. Stevens of the Florida Experiment Station, Gainesville, Florida. Botryotinia Gray Mold (BGM) is a serious disease of castor especially in southern states of India like Andhra Pradesh and Tamil Nadu where the weather conditions are more favorable for disease development where during the year 1987, an epidemic outbreak of gray mold occurred [5].

India ranks 12th position in sunflower production with the yield of 1.41MT (FAO STAT, 2012-13). In India, Sunflower (*Helianthus annuus* L.) is cultivated in around 18 lakh hectares (10% of the world sunflower area) and production is around 12.52 lakh tons (2012-13) (4% of the world sunflower production).

As there is no resistant cultivar available against this disease, it has become inevitable to go for the use of fungicides for the management. Though effective fungicides have been identified for management of BGM and ALB, excessive use of these chemical fungicides may lead to resistance development in pathogen populations. Hence, for control of the diseases one must consider several, rather than just one, management practices. Bio-control agents are a possible alternative to fungicides. There are several studies dealing with the use of biological control agents, mainly Trichoderma spp. to control diseases caused by Botryotinia spp. [7]. There are some studies conducted with *Trichoderma* for the control of gray mold of castor and promising results have been obtained by many researchers [8-12]. However, it is clear that, although promising, these results are still experimental and much work needs to be done before permanent recommendations regarding the use of biological control agents can be secured for gray mold management [13].

Two important criteria in the implementation of a bio-fungicidal mode of bio-control programme are the ability to develop a method for large scale production of the candidate organism and the formulation of its biomass into a commercially acceptable product [14]. The availability of an effective and cheap method to mass produce *Trichoderma* spp. is essential for commercial use of *Trichoderma* as a biopesticide. Several commercial formulations of *Trichoderma* are available in the market (www.apsnet.org/online/feature/Biocontrol). The commonly available formulations of *Trichoderma* spp. include dusts, Pyrex/biomass, alginate pellets, kaolin/starch/cellulose granules and extruded granules [14-17]. Although many formulations for soil incorporation and seed treatment are in the market, not enough inexpensive liquid formulations are commercially available. *Trichoderma* spp. is being extensively marketed as powder formulation in India [18,19]. The problems associated with powder formulations include desiccation of conidia and loss in viability of propagules beyond three to six months of storage [20], which affects efficiency and marketability of the formulation. To overcome some of these problems a Suspension Concentrate (SC) formulation of *Trichoderma* has been developed with long shelf-life for foliar sprays which can support survival of fungal biocontrol agent on foliar parts for longer periods. Intensive research work at laboratory, glasshouse and under field over two seasons on biological control of two diseases viz., Alternariaster leaf blight (ALB) in sunflower and Botryotinia Gray Mold (BGM) in castor to bring out a competent strains and formulation with better persistence on phylloplane and long shelf life is reported here.

Materials and Methods

Isolation of biocontrol agents and pathogens

Isolation of biocontrol agents: The *Trichoderma* species used in this study were isolated from soil samples from different locations of India using Trichoderma Specific Medium (TSM). All the isolates were purified using single spores. Identification of the isolates was confirmed based on morphological characters and molecular methods using oligo-nucleotide barcode (Trichokey). Based on Tricho BLAST (Basic Local Alignment Search Tool) analysis, the isolated bioagents were identified as *T. harzianum* and *T. asperellum* [21]. All the isolates were maintained on Potato Dextrose Agar (PDA) slants at 4°C and thirty potential strains were deposited at National Agriculturally Important Microbial Culture Collection (NAIMCC), National Bureau of Agriculturally Important Microorganisms (NBAIM), Maunath Bhanjan, India and accessions numbers (NAIMCC – F-02032, 37, 39, 42-67; F- 02225-26) have been obtained.

Isolation of *Botryotinia ricini* and *Alternariaster helianthi*: The two pathogens *Botryotinia ricini* (Godfrey) Whetzel and *Alternariaster helianthi* (Hansf.) Tubaki and Nishihara used in the present study were isolated from infected samples collected from experimental fields of Directorate of Oilseeds Research (DOR), Hyderabad, India. *Botryotinia ricini* was isolated using Oat meal agar (OMA) medium. The culture thus obtained was maintained on OMA slants at 4°C. *A. helianthi*, collected from infected samples of sunflower leaf was maintained on Sunflower Leaf Extract Media (SLEM) and stored at 4°C until used.

Preparation of conidia suspension of *T. harzianum* Th4d and *T. asperellum* Tv5: A 7-day old culture of two different species of *Trichoderma* viz., *T. harzianum* Th4d (NAIMCC-F- 02188 and Gene bank accession No. KF471117) and *T. asperellum* Tv5 (NAIMCC-F-02225 and Gene bank accession No. JQ976275) were selected and grown on PDA separately were used to prepare the conidial suspension. Sterile water (10 ml) was added to the culture plates and the surface was scraped lightly with a sterile transfer loop. The resulting suspension was filtered through two layers of sterile muslin cloth. The conidia suspension was adjusted to $1x10^3$ to $1x10^9$ conidia/ml with sterile distilled water using haemocytometer. These conidia suspensions were used for further experiments.

Production of bioagents and development of SC formulations

The Trichoderma spp. were grown on Molasses-Soy Medium (MSM) by inoculation of 5 ml of conidial suspension from sporulating cultures on PDA slants to 250 ml of medium in 500-ml Erlenmeyer flasks. Inoculated flasks were kept in shaking incubator (make Amerex

Instruments Inc., USA) for 3 days at 200 rpm and culture flasks were removed and poured in to the sterile plastic box with dimensions of 36 x 25 x 5 cm. The poured molasses-soya medium is spread uniformly in the tray maintained a depth of 5 mm. The trays with culture were incubated at 25°C for 3 days for profuse conidia production on the surface. The six-day-old dry fungal mat was ground to fine powder. Dry conidial powder was mixed with mineral oil (density–0.838 g/ml at 25°C, viscosity index–102) at 1:3 ratio, stabilizers were added and sedimentation rate or buoyancy was recorded accordingly. The SC formulation was packed in High Density Polyethylene (HDPE) bottles.

Dose standardization of SC formulation for foliar application

Suspension concentrate formulation of bioagents (2×10^9 conidia/ml) was used for foliar spray to determine the effective dosage against *B. ricini* of castor and *A. helianthi* leaf spot of sunflower. Surface sterilized castor hybrid DCH 519 and sunflower variety DRSF 108 were and sown in pots under glass house conditions. Four kilograms of sterile sieved field soil (Red sandy loam; pH 6.1, N, P and K of 185, 22.5 and 195 kg ha^{-1}, respectively) was filled in clean plastic pots (18 × 18 cm).The liquid formulation of two strains of *Trichoderma* isolates namely *T. harzianum* Th4d SC and *T. asperellum* Tv5 SC was sprayed at four different dosages (0.5 ml/l, 1 ml/l, 1.5 ml/l and 2 ml/l). Persistence of *Trichoderma* on sprayed capsules/leaves was determined by serial dilution of washings and plating on to Trichoderma Specific Medium (TSM) at 10^{th} and 20^{th} day after spray. After 24 hours of bioagents spray, spore suspension of B. ricini (7×10^7conidia/ml) and *A. helianthi* (7×10^7conidia/ml) was sprayed in all treatments including the untreated control which served as pathogen check. The first spray of bioagents was given at 35 days after sowing followed by second spray after 10 days in sunflower crop whereas in castor crop, first spray was given when crop was at 80 days old followed by second spray after 10 days. Disease incidence was scored as mentioned below (section 2.4.1) and bioagents population as CFU/ml. The experiment was repeated twice to determine the accurate dosage.

Bioefficacy of SC formulation of *Trichoderma* spp.

In vivo screening of bioagents: A glasshouse pot test was conducted to know the biocontrol potential of twenty five *Trichoderma* spp. against gray mold of castor and Alternariaster leaf blight of sunflower was conducted as in "Dose standardization of SC formulation for foliar application".

Trichoderma isolates were screened for their biocontrol potential against gray mold in castor by detached spike technique in the glasshouse with fogging facility.

Racemes/spikes of 15- day-old were collected from castor plants (var. DCH 519) and were kept in 150 ml conical flasks containing 2% sucrose solution. Racemes were sprayed with bioagents formulation at 2 ml/l and an inoculum (7×10^7 conidia/ml) of Botryotinia was sprayed after 24 hr. Treated racemes were maintained at 25 ± 2°C with 90% humidity, respectively.

Gray mold incidence was scored by determining the percentage of capsules infected on racemes using a 0-9 scale for the gray mold in castor [22]; (0: No infection; 1-1% capsules infected on a raceme; 3-10% of capsules infected; 5-11 to 25% capsules infected; 7-26 to 50% of capsules infected; 9- >50% capsules infected). The Per Cent Disease Index (PDI) was calculated as per Mckinney (1923) infection index.

PDI = (Sum of individual ratings × 100) / (Total number of spikes observed × Maximum disease grade)

To test the efficacy of bioagents against Alternariaster leaf blight in sunflower seeds (var. Morden) were surface disinfected with 2% sodium hypochlorite solution and treated with SC formulation of *Trichoderma* isolates at 2 ml/kg seeds were suspended in appropriate amount of water and seeds were immersed in the suspension and incubated overnight in incubator shaker and spread onto plastic trays for air drying and carbendazim at 2 g/kg treated serve as fungicide control. Three replicates were maintained for each treatment. When sunflower plants were at 35- day-old, first spray of *Trichoderma* (7×10^7 conidia/ml) was given. Spore suspension of *A. helianthi* ($7x10^7$ conidia/ml) was sprayed in all the treatments including the untreated control which served as pathogen check. The second spray of *Trichoderma* isolates was given at 45 days after plant growth respectively. Disease severity of *A. helianthi* in sunflower was recorded using the rating scale of Allen, *et al.* [23].

Field experiments: A field experiment was conducted during kharif 2010 and 2011 to test the efficacy of *T. harzianum* Th4d SC formulation alone and consortium of *T. harzianum* Th4d SC and *T. asperellum* Tv5 SC against gray mold of castor. Field trial was performed in a Randomized Block Design (RBD) with 8 treatments and each treatment replicated thrice. The treatments are T1: *T. harzianum* Th4d SC 1 ml/l, T2: *T. harzianum* Th4d SC 2 ml/l, T3: *T. asperellum* Tv5 SC 1 ml/l, T4: *T. asperellum* Tv5 SC 2 ml/l, T5: *T. harzianum* Th4d SC +T. asperellum Tv5 SC 1 ml/l, T6: *T. harzianum* Th4d SC + *T. asperellum* Tv5 SC-2ml/l, T7: Carbendazim 0.1%, T8: Pathogen control (Botryotinia inoculum sprayed at 10^6 spores/ ml). The plot size was 5.4 × 4.2 m and surface sterilized castor seeds (cv. DCH 519) were soaked in water mixed with SC formulation of bioagent (to treat a kilogram of seed, 2 ml of SC formulation mixed in 1 litre of water and seed is immersed in the bioagent suspension and incubated overnight in by periodic shaking). The soaked seeds were air-dried and sown with a spacing of 90 x 60 cm.

Congenial conditions for disease development were created by using fogging devices. For maintaining capsule wetness and humidity in the field, four way foggers were fixed on PVC lateral pipes drawn at a height of 6 ft one line each between two castor rows from underground irrigation pipelines for which pre-filtered (30 m^3 screen filter) irrigation water is supplied at a pressure of 4 kg/cm^2 from water source. When castor plants are at an age of 80 days with 2-3 spikes/ racemes of 15-20-days-old, bioagents spray was taken and fogging system is operated every 1 hr for 10 min during day time so that capsule remain wet at least for 6-8 hrs/day upto 20 days. Botryotinia was sprayed (10^7 spores/ml) two days after bioagents treatment. Chemical fungicide (carbendazim, 0.1%) spray was taken up one day before pathogen inoculation. Two sprays of bioagents and chemical fungicides were given at weekly intervals. Spore suspension (10^7 spores/ml) of the Botryotinia was sprayed at 15 days interval after first spray to infect young castor spikes/racemes. *B. ricini* conidiospore suspension (10^7 spores/ml) prepared from 10-day-old culture grown on OMA medium was used. Ten plants from each replicated treatment were randomly selected and tagged for grading the disease incidence and seed yield. The disease incidence was recorded as mentioned above in "In vivo screening of bioagents".

Survival of bioagents on castor spikes/racemes: The survival and persistence of SC formulation of *Trichoderma* on the racemes /spikes of castor were determined by collecting the capsules immediately after spray, at 7 days and 14 days after spray on castor (var. DCH-519)

racemes/spikes in the field. The initial concentration of *Trichoderma* in spray fluid was 10^7 CFU/ml. In each treatment, 5 racemes /spikes were selected randomly and capsules from each racemes /spike were kept in sterile distilled water and kept on shaker at 200 rpm for 10 min. Serial dilutions from these samples were plated with 3 replications on TSM for enumeration of *Trichoderma* isolates. The plates were incubated at 28°C and number of CFU of *Trichoderma* counted after 48 and 96 hr, respectively. Survival of bioagents on racemes/spikes expressed as log CFU/g of capsules.

Field evaluation of SC formulation of *T. harzianum* Th4d alone and consortia of *T. harzianum* Th4d and *T. asperellum* Tv5 bioagents against Alternariaster blight of Sunflower: Field trials was conducted during 2010 and 2011 at DOR in a RBD with 8 treatments and 3 replications were maintained for each treatment. The plot size was 5.4 x 4.2 m and sunflower (var. Morden) seeds were surface sterilized and treated with SC formulation of bioagents as explained in section 2.4.3 and sown with a spacing of 60 x 30 cm. The treatments are T1: *T.harzianum* Th4dSC1ml/l,T2: *T.harzianum* Th4dSC2ml/l,T3: *T.asperellum* Tv5SC1ml/l,T4: *T.asperellum* Tv5SC2.0ml/l,T5: *T.harzianum* Th4dSC+ *T.asperellum* Tv5SC1ml/l,T6:T.harzianumTh4d+ *T.asperellum* Tv5-2ml/l, T7: Carbendazim 0.1%, T8: Pathogen control (Alternariaster inoculum sprayed at 10^6 spores/ml). Bioagents and chemical fungicide spray were given at 35 days after sowing followed by pathogen spray after 24 hr. The second spray of bioagents and fungicides was given at 15 days after first spray. Ten plants from each replication were randomly selected and tagged for grading the disease severity and seed yield. Disease severity was recorded using the rating scale of Allen, *et al.* [23].

Persistence of biocontrol agents on leaves of sunflower: The survival and persistence of bioagents on sunflower leaves was determined by collecting leaves immediately after first spray, at 7 and 14 days after the first spray on sunflower (var. Morden) leaves in the field. The initial concentration of *Trichoderma* in spray fluid was 10^7 CFU/ml. In each treatment, 5 plants were selected randomly and 5 leaves from each plant were used for estimation of *Trichoderma*. Leaves were washed in sterile water and washings were serial diluted and used for determination of CFU as explained in section "Survival of bioagents on castor spikes/racemes".

Assay of induced enzymes: Induction of defense related enzymes in castor and sunflower was studied by selecting the two potential strains of *Trichoderma* SC formulations (*T. harzianum* Th4dSC and *T. asperellum* Tv5SC) which were found effective against gray mold of castor and Alternariaster leaf blight of sunflower under glass house conditions.

The castor (cv. DCH 519) and sunflower (var. Morden) seeds were surface disinfected with 2% sodium hypochlorite solution and treated with SC formulation of Th4d and Tv5 at 2 ml/kg seeds were suspended in appropriate amount of water and seeds were immersed in the suspension and incubated overnight in incubator shaker and spread onto plastic trays for air drying and untreated served as check. Three replicates were maintained for each treatment. When sunflower plants were at 15-day-old, first spray of *Trichoderma* (7×10^7 conidia/ml) was given and second spray of *Trichoderma* isolates was given at 30 days after plant growth respectively. Similarly in castor the first and second spray were given when plant was at 15 and 30 day old. Leaf tissues from sunflower and castor was collected at different time intervals (0, 24, 48, 72, 96 and 164 hr) after each spray and quickly frozen in liquid nitrogen and stored at -20°C.

For assay of Peroxidase (PO), Phenylalanine Ammonia Lyase (PAL) and Polyphenol Oxidase (PPO) activity one gram fresh weight of leaf tissue were ground to a fine powder in liquid nitrogen and extracted in 1 ml of extraction buffer containing 0.1 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 15,000 g for 15 min at 48°C and the supernatant was used as enzyme source to estimate PO, PAL and PPO activity as per standard protocols followed by Govindappa, *et al.* [24].

Viability of *Trichoderma* in stored formulations (Shelf-life)

The viability of SC formulation of *T. harzianum* Th4d SC and *T. asperellum* Tv5 SC stored in HDPE bottles at two different temperature viz., 4°C and 30 ± 2°C was determined at bimonthly intervals till 18 months. The number of colony forming units was determined by serial dilution and plating it on *Trichoderma* Specific Medium (TSM) [25] and incubating petri plates at 25 ± 2°C.

Statistical analysis: *In vivo* and field experimental data was subjected to analysis of variance (ANOVA). Data in percent were arcsine transformed before analysis. The treatment means were compared by Duncan's Multiple Range Test (DMRT) [26].

Results

Dose standardization of Trichoderma SC formulation for foliar application

Four different concentrations of SC formulation of *Trichoderma* viz., 0.5 ml/l (9×10^4), 1 ml/l (18×10^5), 1.5 ml/l (4×10^6) and 2 ml/l (6×10^7) were screened against gray mold of castor and Alternariaster leaf spot of sunflower. *T. harzianum* Th4d SC at 2 ml/l with effective dosage of 2×10^7 (Figure 1a and 1b) is considered as effective control of diseases and this dosage has been considered as standard for further screenings.

In vivo screening of bioagents

Effect of Trichoderma isolates in reducing the gray mold of castor and Alternariaster leaf blight of sunflower: A total of 25 Trichoderma strains were screened in vivo against gray mold of castor and Alternariaster leaf blight of sunflower (Table 1).

Among different strains of *Trichoderma* tested, *T. harzianum* Th4d and *T. asperellum* Tv5 were found to be effective in disease reduction of gray mold of castor and Alternariaster leaf blight of sunflower.

Among all the treatments *T. harzianum* Th4d treatment was found to be most effective against gray mold of castor recorded lowest disease severity (13.4%) with a disease reduction of 79.0 % compared to pathogen check. *T. asperellum* Tv5 was found to be equally effective against gray mold of castor with a disease reduction of 70.6%. All other bioagents treatment recorded disease reduction from 68.0 to 21.3%.

Similarly *T. harzianum* Th4d treatment was found to be effective against Alternariaster leaf blight of sunflower recorded lowest disease severity (23.5%) with a disease reduction of 63.5% compared to pathogen check. *T. asperellum* Tv5 was found to be equally effective against Alternariaster leaf blight of sunflower with disease reduction of 60.5%. All other bioagents treatment recorded disease reduction from 59.7 to 11.1%.

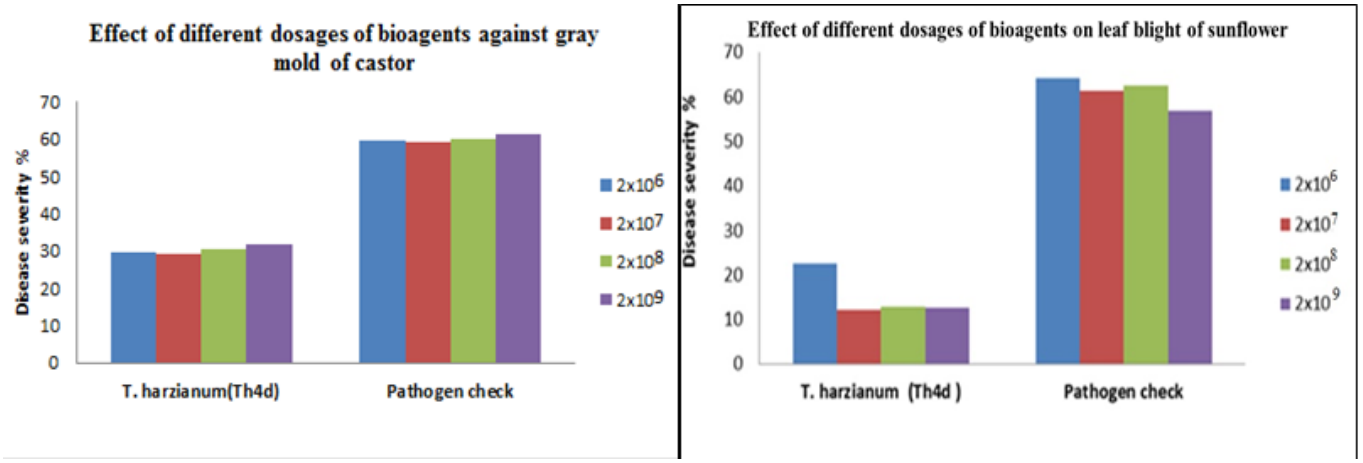

Figure 1: a) The antagonistic effect of Trichoderma isolates at different conidia concentrations against Botryotinia gray mold of castor, b) The antagonistic effect of Trichoderma isolates at different conidia concentrations against A.helianthi of sunflower

Name of Bioagents	Gray mold of castor		Leaf blight of sunflower	
	Disease severity (%) *	Disease reduction over control (%)	Disease severity (%) *	Disease reduction over control (%)
T. asperellum (Tv3)	22.4^{l}	65	25.8^{abc}	59.7
T. asperellum (TvN13)	20.7^{lmn}	67.7	27.6^{gi}	56.9
T. asperellum (Tv33)	21.2^{lm}	66.9	31.9^{gi}	50.2
Trichoderma spp. (T12)	19.2^{n}	70	27.6^{fg}	56.9
Trichoderma spp. (TS4)	36.4^{f}	43.2	31.9^{gi}	50.2
Trichoderma spp. (TS7)	50.5^{b}	21.3	53.2^{cdefgi}	17.2
Trichoderma spp. (TS9)	50.0^{b}	22.2	57.1^{a}	11.1
T.harzianum (TS10)	42.8^{d}	33.3	46.4^{ab}	27.8
T. harzianum (TS12)	23.0^{lk}	64.1	30.7^{bcdefg}	52.2
Trichoderma spp.(T8)	28.9^{h}	55	53.2^{cdefgi}	17.2
T. asperellum (Tv5)	18.8^{n}	70.6	25.4^{bcdefi}	60.5
T. harzianum (Th4d)	13.4^{o}	79	23.5^{i}	63.3
T. asperellum (Tv7)	20.5^{lm}	68	35.7^{i}	44.4
Trichoderma spp. (TSP3)	39.1^{e}	39	50.0^{bcdefg}	22.2
Trichoderma spp. (TA5)	51.4^{b}	20	46.4^{abcd}	27.8
Trichoderma spp. (TS3)	46.4^{c}	27.8	53.2^{abcd}	17.2
T asperellum (Tv22)	53.2^{a}	17.2	50.0^{abc}	22.2
T. asperellum (Tv11)	36.6^{f}	43	42.8^{bcd}	33.3
T. asperellum (Tv2)	19.8^{mn}	69.1	26.0^{defgi}	59.4
T. asperellum (Tv12)	33.5^{g}	47.8	43.5^{fgi}	32.3
T. asperellum (Tv8)	43.5^{d}	32.3	48.2^{bcde}	24.8

Trichoderma spp. (T4)	42.8^{d}	33.3	50.0^{abcd}	22.2
T. asperellum (Tv6)	27.1^{i}	57.7	33.2^{abcd}	48.3
T.harzianum (Th7)	24.2^{j}	62.2	36.4^{cdefgi}	43.2
T. asperellum (TSP1)	19.8^{mn}	69.1	30.7^{defgi}	52.2
Carbendazim – 0.1%	23.0^{k}	64.1	28.9^{efgi}	55
Pathogen check	53.5^{a}	16.6	64.2^{gi}	-
SEm.±	1	-	0.07	-
CD (P= 0.05)	1.2	-	2	-
CV (%)	2.2	-	3	-

Table 1: Screening of *Trichoderma* strains against gray mold of castor and Alternariaster leaf blight of sunflower. *Mean values presented are arcsine transformed and the Mean values denoted by the same letter within columns are not significantly different by Duncan Multiple Range Test (P= 0.05), analyzed through ANOVA.

Field evaluation trials

Field evaluation of bioagents against gray mold of castor (2009-10 and 2010-11): The intensity of B. ricinni and seed yield were significantly influenced by different seed treatments during both the years (Table 2).

Treatments	2009-10 & 2010-11		
	Disease severity (%)*	Reduction over pathogen check (%)	Seed yield (Kg/ha)
T1: *Trichoderma harzianum* Th4d SC - 1ml	29.9^{bc}	54.8	1610^{a}
T2: *Trichoderma harzianum* Th4d SC - 2ml	29.2^{cd}	55.8	1737^{a}
T3: *Trichoderma asperellum* Tv5 SC - 1ml	32.1^{b}	51.4	1712^{a}
T4: *Trichoderma asperellum* Tv5 SC - 2ml	32.0 ^{b}	51.9	1513^{b}
T5: *T.harzianum*Th4d+*T. asperellum*Tv5 SC -1ml	25.0^{ef}	61.6	1757^{a}
T6: *T. harzianum* Th4d + *T. asperellum* Tv5 SC -2 ml	26.5^{de}	58.6	1637^{a}
T7: Carbendazim 0.1%	34.5^{f}	50.2	1271^{b}
T8: Pathogen check	63.9^{a}		832^{c}
SEm±	1.5		90.4
CD (p=0.05)	3.3		193.9
CV (%)	5.7		7.7

Table 2: Efficacy of SC formulation of bioagents against gray mold of castor (pooled means 2009-10 and 10-11). *Mean values presented are arcsine transformed and Mean values denoted by the same letter within columns are not significantly different by Duncan Multiple Range Test (P= 0.05), analyzed through ANOVA.

On the basis of two years field trials, *T. harzianum* SC Th4d at 2 ml/l and *T. harzianum* Th4d+*T. asperellum* Tv5 SC at 1 ml/l was observed to be equally effective with carbendazim 0.1% against gray mold of castor with maximum disease reduction over pathogen check and resulted in higher yield when compared to carbendazim and pathogen check (Table 2).

Among all the individual bioagents treatment *T. asperellum* Th4d SC at 2 ml/l treatment was found to be effective against gray mold of castor and recorded lowest disease severity (29.2%) with a disease reduction of 55.8% compared to pathogen check. *T. harzianum* Th4d + *T. asperellum* Tv5 SC at 1 ml/l was found to be equally effective against gray mold of castor with disease reduction of 61.6%. All other bioagents treatment recorded disease reduction from 59.1% to 47.4%. *T. harzianum* SC Th4d at 2 ml/l and *T. harzianum* Th4d + *T. asperellum* Tv5 SC at 1 ml/l resulted in higher yield (1737 kg/ha and 1757 kg/ha) when compared to pathogen check (832 kg/ha).

Field evaluation of bioagents against leaf blight of sunflower (2009-10 and 2010-11): The intensity of Alternariaster leaf blight of

sunflower and seed yield were significantly influenced by different seed treatments during both the years (Table 3).

Among different treatments, *T. harzianum* SC Th4d at 2 ml/l and *T. harzianum* Th4d+*T. asperellum* Tv5 SC at 1 ml/l was observed to be equally effective with carbendazim 0.1% against leaf blight of sunflower with maximum disease reduction over pathogen check and resulted in higher yield when compared to carbendazim and pathogen check.

Treatments	2009 and 2010		
	Disease severity (%)*	Reduction over pathogen check (%)	Seed yield (kg/ha)
T1: *Trichoderma harzianum* Th4d SC – 1 ml	29.6^{bc}	49.3	1469.5^{c}
T2: *Trichoderma harzianum*Th4d SC – 2 ml	27.8^{bc}	50.9	1618^{bc}
T3: *Trichoderma asperellum* Tv5 SC – 1 ml	29.9^{b}	47.8	1546^{c}
T4: *Trichoderma asperellum* Tv5 SC – 2 ml	27.8^{c}	53.2	1491^{c}
T5: *T. harzianum*Th4d+*T. asperellum* Tv5 SC -1 ml	21.8^{d}	61.7	1757^{c}
T6: *T. harzianum*Th4d+*T. asperellum*Tv5 SC -2ml	22.9^{d}	59.3	1637^{b}
T7: Carbendazim 0.1%	34.9^{ad}	45.3	1271^{d}
T8: Pathogen check	57^{a}	-	832^{e}
S. Em ±	1.4		58.9
CD (p=0.05)	3		126.5
CV (%)	6.3		4.7

Table 3: Field evaluation of Potential bioagents against Alternariaster blight of sunflower (pooled means 2009-2010 and 10 -2011). * Mean values presented are arcsine transformed and Mean values denoted by the same letter within columns are not significantly different by Duncan Multiple Range Test (P= 0.05), analyzed through ANOVA

Among all the individual bioagents treatment *T. harzianum* SC Th4d at 2 ml/l was found to be effective against leaf blight of sunflower recorded lowest disease severity (27.8%) with a disease reduction of 50.9% compared to pathogen check. *T. harzianum* Th4d+*T. asperellum* Tv5 SC at 1 ml/l was found to be equally effective against leaf blight of sunflower with disease reduction of 61.7%. All other bioagents treatment recorded disease reduction from 53.5 to 39.7%. *T. harzianum* SC Th4d at 2 ml/l and *T. harzianum* Th4d+*T. asperellum* Tv5 SC at 1 ml/l resulted in higher yield with 1618 kg/ha and 1757 kg/ha when compared to pathogen check (832 kg/ha) respectively.

Survival of bioagents on castor spikes and persistence on sunflower leaves: Varied concentrations of SC formulation of *Trichoderma* alone and *T. harzianum* Th4d SC+ *T. asperellum* Tv5 SC combinations were able to survive and maintain their population on sprayed capsules of castor and on leaves of sunflower in good numbers up to 14 days (Figure 2).

Among all the treatments *T. harzianum* Th4d SC at 2 ml/l and *T. harzianum* Th4dSC + *T. asperellum* Tv5 SC at 1 ml/l showed better persistence up to 14 days.

Assay of Defense enzymes

Increased activity of defense related enzymes viz., Peroxidase (PO), Polyphenol Oxidase (PPO) and Phenylalanine Ammonia-Lyase (PAL) activity in *T. harzianum* Th4d SC and *T. asperellum* Tv5 SC was observed in pre- treated castor and sunflower plants. Significant accumulation of PO, PPO and PAL activity at 30 days was high compared to15 days in these bioagents. The activity of PO reached the highest level in plants at 48 hr after treatment with *T. harzianum* Th4d SC and then slowly decreased (Figure 3).

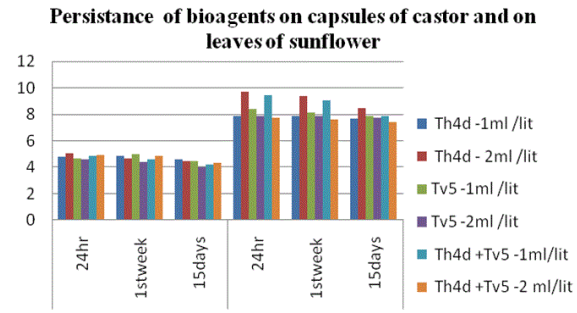

Figure 2: Persistence level of SC formulation of *Trichoderma* on the phylloplane of sunflower and persistence on castor spikes was examined at weekly intervals

Similarly PPO activity increased significantly at 30 days in bioagents compared to control (Figure 4). PAL activity reached maximum at 48 hr of inoculation (Figure 5).

Shelf life of the formulation

The population count (CFU) was found to be highest in all the SC formulations of *T. harzianum* Th4 SC and *T. asperellum*Tv5 SC for 18 months period (Table 4). At storage temperature of 4°C, *T. harzianum*

Th4d SC + *T. asperellum* Tv5 SC retained significantly high population (log 6.88) up to 18 months period but at 30 ± 2°C the bioagent population declined was not significant up to 14 months and there after population declined significantly.

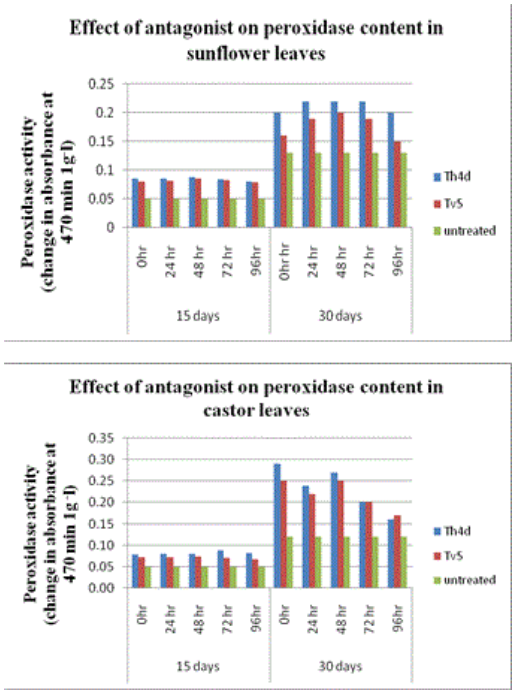

Figure 3: Induction of peroxidase in sunflower and castor response to biocontrol agents treatment.

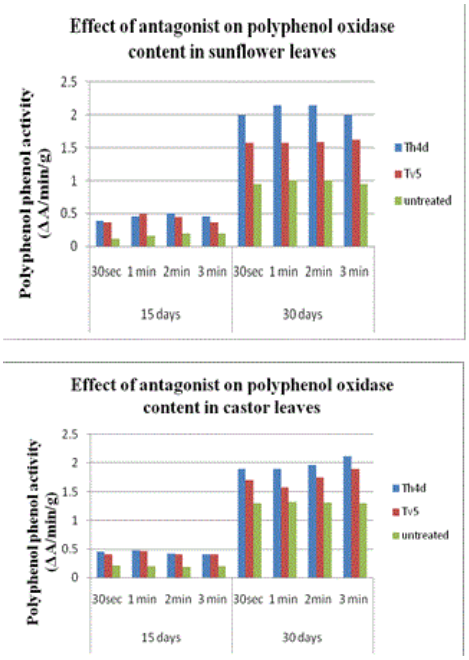

Figure 4: Induction of PPO activity in sunflower and castor treated with biocontrol agents.

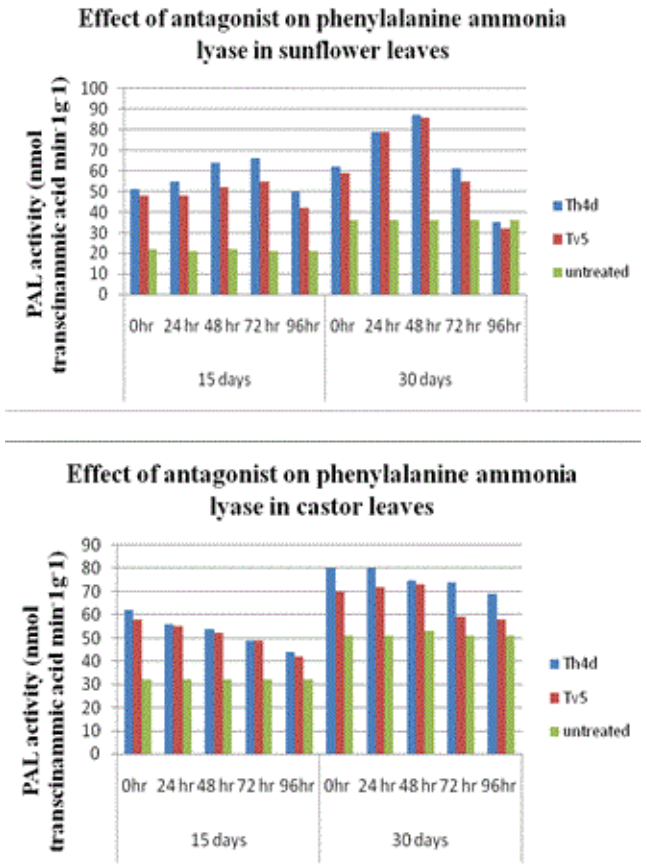

Figure 5: PAL activity in sunflower and castor in response to biocontrol agents treatment.

Discussion

Based on the results obtained the optimum concentration of *Trichoderma* spp. SC at 2 ml/l with effective dosage of 2×10^7 conidia/ml was found to be effective in control of ALB of sunflower and gray mold of castor and this dosage is used for further screenings. The concentration of conidia required is rather high; hence, the mass production of the antagonist to obtain the required conidia by a rapid efficient and inexpensive fermentation is a vital requirement.

The efficacy of talc – based BCAs has already been demonstrated on foliar diseases and few commercial BCAs formulations are developed aiming at foliar pathogens in countries like USA [27], Italy [28] and Israel [29]. In India BCAs were tested against foliar pathogens with variable performances under varied environmental conditions in the field. This is mainly due to improper selection of candidate strains which are not phylloplane competent and failed to establish an active population on the phylloplane; however, the carrier based bio-inoculants suffer from short shelf life, high contamination and low field performance [30].

In the present investigation a liquid formulations of *Trichoderma* spp. were developed using locally available oils (mineral oil), emulsifiers and stabilizers to overcome some of the inherent problems associated with powder based formulations of *Trichoderma*.

Time (months)	Log CFU			
	T. harzianum Th4dSC		*T. asperellum* Tv5SC	
	4°C	30 ± 2°C	4°C	30 ± 2°C
1	11.17	11.17	11.3	11.3
2	11	10.75	11	10.8
4	10.65	10.44	10.85	10.65
6	10.32	10.1	10.6	10.35
8	10.2	9.8	10.4	10.1
10	10.15	9.76	10.35	10.06
12	9.91	9.54	10.24	10
14	9	8.98	10.1	9.55
16	8.45	8.23	9.52	9
18	8	7.4	8.8	7.6

Table 4: Spore viability (Log CFU's) of SC based formulation of *T. harzianum* Th4d SC and *T. asperellum* Tv5 SC stored at room temperature (30 ± 2°C) and at 4°C in HDPE bottles.

SC formulation was developed using conidial biomass of these bioagents by following a production process supporting a higher quantities of biomass and viable propagules. A patent for process of "Production process for improved yield of *Trichoderma* Biomass" has been filed and published (568/CHE/2012A). The formulation thus prepared readily disperse in water to form a stable emulsion, is easy to handle and possess good bioefficacy with better adhesion and penetration on target site leading to control of foliar pathogens and assumes greater importance in sustainable crop protection. The formulation used in this study showed no undesirable side effects like phytotoxicity to leaves and on the quality of castor and sunflower seeds. Since the formulation ingredients, especially oil and emulsifier are commonly used as food additives and in the manufacturing of cosmetics; they can be considered environmentally and toxicologically safe.

SC formulation of *T. harzianum* Th4d SC and *T. asperellum* Tv5 SC had better persistence on the phylloplane up to 15 days (Figure 2) which help in success of biological control on foliar surfaces. These isolates found to be efficient in solubilizing inorganic phosphate and production of mycolytic enzymes in our earlier work [31].

Based on our previous findings, *T. harzianum* Th4d SC and *T. asperellum* Tv5 SC treated in castor and sunflower plants showed penetration of fungal hyphae in to the plant roots, conization of the root epidermis and cortex cells but not the vessels at early stages of castor and sunflower root growth [32]. A similar behavior was reported for cucumber roots after inoculation with *T. harzianum* strain T-203, i.e., extensive colonization of the root surface, appressoria-like structures, and hyphal filaments penetrating the root epidermis between adjacent cells, generating a lytic zone around the penetrated area [33].

Further from this study it has been concluded that seed treatment and spray on racemes/spikes of castor and on leaves of sunflower with

SC formulation of *Trichoderma* induced significant protection against foliar diseases. The possible mechanism involved was direct antagonism, effective root colonization and persistence competency leading to the induced systemic resistance.

Other than direct action, these biocontrol agents triggered defense related enzymes involved in phenyl proponoid pathways, higher activity of peroxidase, phenylalanine ammonia-lyase, polyphenol oxidase was observed with (*Trichoderma* Th4d SC and *T. asperellum* Tv5 SC) these bioagents when compared to untreated control with castor and sunflower plants. Seed treatment and foliar application with these bioagents could be very effective in the control of gray mold of castor and leaf blight of sunflower with better seed yield compared to pathogen check and they also induced systemic resistance and physiological changes leading to plant defense mechanisms.

These findings are in agreement with those of several workers. Bio formulation of *T. virens* sprayed on leaves and flowers increased the induction of peroxidase activity in cucumber [34]. High peroxidases activity was observed by treatment with *P. fluorescens* and *T. harzianum* followed by *B. subtilis* over control are associated with stages of the infection process and are involved in generation of hydrogen peroxides it inhibit the pathogen directly by producing free radicles with antimicrobials effects and lignifications [35]. PAL accumulation was high at 48 h after challenge inoculation and the PAL is a product of cinammic acid; it is directly linked with lignifications. Our results confirm the findings of Podile and Laxmi [36] and Silva, *et al.* [37]. In host–pathogen interactions increased levels have been shown to be correlated with incompatibility [38]. PPO activity is also enhanced due to treatment with biocontrol agents and it catalyses the last step of biosynthesis of lignin and other oxidative phenols.

Intensive research has been carried out to bring out the potential biocontrol strains against Botryotinia gray mold management from our prior work [39-42]. Among the varied concentrations SC formulation evaluated, *T. harzianum*Th4dSC 2 ml/l and *T. harzianum*Th4d SC + *T. asperellum* Tv5SC 1 ml/l was observed to be effective against *A. helianthi* of sunflower and Botryotinia gray mold of castor. *Trichoderma* isolates were found to be effective against gray rot of rose [43]. *T. harzianum* is an effective antagonist against *B. cinerea* on strawberry and apple fruits in emulsion formulations [44]. Several researchers conducted studies using *Trichoderma* and *Clonostachys rosea* for control of gray mold of castor and effective results were obtained [8-12,45]. In controlling early and late leaf spot of blight in sunflower, *T. viride* was found to be equally effective as that of fungicides [46].

Even after 18 months of storage, at ambient temperature, SC formulation of *Trichoderma* (Th4d and Tv5) showed good shelf life prepared with mineral oil with addition of an emulsifier and stabilizer in appropriate ratio increased the desiccation tolerance of propagules in the formulation without reducing the CFU of *Trichoderma* on prolonged shelf life. Kolombet, *et al.* [47] reported the extended shelf life of *T. asperellum* in liquid formulation. Further studies of the impact of slowly available nutrient sources and metabolic inhibitors to prolong the shelf-life of SC based formulations of fungal biomass appear to be justified. Modification of the application methods or supplementation of formulations with additives may enhance the performance of the biocontrol agents on the phylloplane [48]. The use of fungal BCA's that have been formulated for inundative application has been extensively studied but only a few products are commercially available [49].

Thus SC formulation of *T. harzianum* Th4d SC and *T. asperellum* Tv5 SC formulations have a good rate of viable propagules upto 18 months of storage, at ambient temperature, *T. harzianum* Th4d SC 2 ml/l and consortia of *T. harzianum* Th4d + *T. asperellum* Tv5 SC 1 ml/l resulted maximum disease reduction with a better persistence rate upto 2 weeks and resulted in higher yield compared to pathogen check, which lead to successful protection from gray mold of castor and leaf blight of sunflower. The use of SC formulation in the management of several other diseases is under progress for further commercialization process.

Acknowledgments

This work was financially supported by the ICAR Network - Applications of Microorganisms in Agriculture and Allied sectors (AMAAS) project and sincere thanks to Project Director, Directorate of Oilseeds Research (DOR) for providing the facilities required for conducting this study.

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