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Efficacy of Fungicides and Culture Filtrate of Native Bioagents against *Didymella bryoniae* Causing Gummy Stem Blight Disease in Gherkin

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ABSTRACT

Fungicides and culture filtrate of native bio-agents were evaluated to test their proficiency to control gummy stem blight disease caused by Didymella bryoniae in Gherkins. Amongst the tested culture filtrate of antagonistic bioagents, Trichoderma harzianum (THRHG1 strain) at 20% concentration showed 43.1% mycelial growth inhibition which was maximum followed by Penicillium purpurogenum -1 (PPDMK1) at 5% concentration which showed 3.36% growth inhibition. Aspergillus terreus (ATKGM1) and Penicillium purpurogenum-2 (PPDMK-2) showed minimum growth inhibition. Further, Didymellq bryoniae showed highly sensitive to fungicides viz. Mancozeb 75% (Indofil) and Tebuconazole 50% (Nativo) with 100% growth inhibition at very low concentration and found to be least sensitive to other fungicides viz. Allite, Sectin and Kavach. In green house experiments A. terreus (ATKGM1) and P. purpurogenum-2 (PPDMK-2) significantly reduced the disease incidence by 43.8% and 44.6% respectively, when compare to negative control which showed 96.5% disease incidence. Furthermore, both isolates were proved to be good growth promoter compared to overall control which was evident by increase in shoot length 54.2±7.9 and 62.0±8.3, number leaf 13.5±1.0 and 11.1±1.8 respectively. Interestingly, T. harzianum, which was highly effective in vitro, proved to be less effective in green house condition. These results revealed A. terreus and P. purpurogenium can become a good biocontrol agent in managing gummy stem blight disease caused by D. bryoniae in Gherkins.

Key words: *Bioagents, Didymella bryoniae, Fungicides, Gummy stem blight, Poisoned food technique.*

INTRODUCTION

Gherkin is perennial short duration crop, cultivated only by contract farming in India. The crop belongs to the family Cucurbitaceae commonly called pickling cucumber or mini cucumber (*Cucumis sativius* Linn.) usually used for salads, pizza topping, sandwiches and it is major dietary constituent of Eastern and European countries. The crop is mainly grown for export purpose due to low domestic demand in Indian market.

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Cultivation and processing is begins in 1990 extend to Karnataka, Andhra Pradesh and Tamil Nadu. Later the production has been extended to other districts across the state. India exported 180820.87 MT of Cucumber and Gherkins, worth Rs. 942.72 crores (141.02 USD Millions) during the year 2016-17 with major destinations being USA, Belgium, Russia, France and Spain[1]. At present gherkin cultivated in 20 districts in Karnataka. There are about 200 diseases reported in cucumber and Gherkins, caused by various phyto-pathogens leads to crop loss by reducing income of growers. Among them Gummy stem blight (GSB) is an important disease caused by *Didymella bryoniae* (Auersw) Rehm (syn. *Stagonosporosis spp.*), is an Ascomycetes fungus which infects about 12 genera and 23 species of cucurbits[2]. It has been reported that two *Stagonosporopsis* species are known to cause gummy stem blight of cucurbits posing threats to gherkin productivity and other cucurbits in India[3]. The disease can occur at any point in plant growth from seedling stage to fruiting stage. The fungus persists in crop residue even at extreme temperature such as -9^0C for 14 days[4]. The name gummy stem blight is used when leaves and stems are infected, if the organism infects the fruit it is known as black rot disease. In initials stages of disease the fungus cause pale brown lesion on leaves and stem part. Lower part of stem cracks leads to splits, get open and release amber colour gummy sap with black pycnidia on the base of stem and leaves under sever condition, rain or heavy dew leads to release of ascospores and spread as primary inoculums.

Physical control like drenching and chemical control by fungicides like benomyl, chlorothalonil, triforin, zineb, etc., which are used successfully to control the gummy stem blight diseases on other species of cucumber. However, high volume of fungicide spraying leads to fertility loss in the soil and certain biochemical changes can also take place, such as, change in total sugar, phenolic compounds and accumulation of enzymes[5]. Further, continuous use of fungicides may lead to the appearance of new resistant strains of pathogens. Plant based products like crude extracts and essential oils of *Achillea millefolium, Cymbopogon citrates, Eucalyptus citriodora* and *Ageratum conyzoides* were also used in the management of disease caused by *D. bryoniae* with little success. Further, *D. bryoniae* can be considered a high-risk pathogen for the development of resistance because it has a short life cycle and abundant sporulation which, in turn, demands a frequent application of fungicides for its management. However, frequent use of fungicide lead to the development of new *D. bryoniae* isolates that were insensitive to fungicides as reported from Georgia, North Carolina, South Carolina, Delaware, and Maryland in USA[6,7,8,9].

Utilization of fungal biocontrol agents is one of the unconventional and successful methods for manage phytopathogens as previously formulated and applied to control various diseases. Bioagents control the pathogen by antagonstic mechanism of action like mycoparasitism, antibiotics or enzymes production, competition which offers best selection of effective measures for utilization of bioagents and hence, bio-control agents are promising organism for controlling different plant pathogen. However, very meagre information is available on the use of bio-agents against *D. bryoniae* as not much work has been carried out.

As *D. bryoniae* is considered a high-risk pathogen for the development of resistance because it has a short life cycle and abundant sporulation which, in turn, demands a frequent application of fungicides for its management which leads to development of new strains of *D.bryonia*[6,7,8,9]. Hence, the aim of the study was to evaluate the efficacy of fungal bioagents against *D. bryoniae* by conducting *in vitro* studies and also to study the efficacy of synthetic fungicides against *D. bryoniae* for the selection of most prominent bio-agents and fungicide which was effective

at possible minimal concentration to manage the disease.

MATERIAL AND METHODS

Field survey was carried out to study the disease severity and also to collection the disease samples in different part of southern Karnataka. The diseased samples thus collected were brought to laboratory and processed for isolation of the causal agent.

Isolation, purification and characterization of *D. bryoniae*

The infected plant parts were washed and surface sterilized with 1% sodium hypochloride, 0.01% Mercuric chloride solution and then with 70% alcohol. After each treatment samples were rinsed with sterilised double distilled water for 5 min. Then samples inoculate to sterile petri plates containing Potato Dextrose Medium (PDA) and incubated for 7 days at 24°C under 12hrs photoperiod. Identification of pathogen was carried out with suitable keys based on morphological characters. The pathogen was re-isolated from colonies and sub cultured on PDA. Pure culture was maintained for further studies.

Isolation of gherkin rhizosphere mycoflora

Serial dilution method was followed to isolate native bio-agents from gherkin rhizosphere soil samples collected from gherkin growing fields in southern Karnataka state. Solution from appropriate dilution was spread on the surface of PDA medium and incubated at 24°C for 5-6 days. Representative colonies growing on plates were selected, isolated and kept on the same sources media till further use.

Effect of non-volatile (culture filtrate) compounds of bioagents on the growth of *D. bryoniae*

Culture filtrate method was adopted for determining efficacy of non-volatile substances[10]. The isolates of bio-agents were inoculated in 100ml sterile potato dextrose broth in 250ml conical flasks and incubated at 24°C(±1) on a rotary shaker at 150 rpm for 7 days. After 7 days, culture filtrates were filtered through cellulose acetate syringe-filter (0.2µm) and mixed in molten PDA medium (40°C) at different volume to obtain a final concentration of 5%, 10%, 15% and 20% (v/v). The culture filtrate inoculated media was poured into the petri plates at 10ml per plate in three replicates and 5mm discs agar plugs of pathogen was transferred to this media after solidification. Control plates was maintained without amending the culture filtrate and incubated at 24°C (±1) for 7 days. Radial growth of pathogen was recorded and percent inhibition was calculated by using the following formula[11].

$$PI = \frac{C - T}{C} \times 100$$

Where, PI = Percent inhibition.

C = Growth of pathogen in control plate.

T = Growth of pathogen in treatment plate.

Evaluation of fungicides by poison food technique

The efficacy of five commercial namely, Mancozeb 75% (Indofil), Mancozeb 50% (Sectin), Tebuconazole 50% (Nativo), Chlorothaloni tech 78.12% (Kavach) and Fosetyl-aluminium 80% (Allite) were assayed *in vitro* against *D. bryoniae* at different concentration viz. 0.025%, 0.05%, 0.075% and 0.01% by poison food technique. Radial growth was measured when the pathogen attained maximum growth in control plates. The efficacy of the fungicides was expressed as percent inhibition of mycelial growth over control. The percent growth inhibition was calculated by the above mentioned formula.

Green house experiment

Mass production of bioagents and pathogen

The sorghum seeds (100g) were soaked in distilled water for overnight in 250mL conical flask, boiled in a hot water bath at 100 °C for 10 minutes, filtered to remove water and sterilized in an autoclave. The sterilized seeds were kept in laminar air flow for cooling and 8-10 seeds were spread in petri plate containing previously inoculated mycelial mat of pathogen. Same procedure was followed to obtain the bioagents as well. Then the plates were incubated for mass production till the fungal growth completely covered the seed and / or for 10 days. Sorghum seeds over-

grown with pathogen and / or bioagents were used as inoculum for pot experiment.

Pot experiment

Pot experiments were conducted at Botanical Garden, Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore, Karnataka. The soil collected from garden was cleaned, air dried and dry heat sterilized in hot air oven for 48hrs at 120°C. The sterilized soil was amended with pathogen and bioagents at the concentration of 5% w/w. The experimental set up was as below:

Treatment 1: Over all control (no pathogen and no bioagent)

Treatment 2: Negative control (pathogen only)

Treatment 3: THRHG1 isolate + Pathogen

Treatment 4: ATKGM1 isolate + Pathogen

Treatment 5: PPDMK1 + Pathogen

Treatment 6: Positive control (Fungicides + Pathogen)

The sterilized soil was amended with respective treatments, mixed thoroughly and left for one week before seed sowing. Each pot was seeded with five seeds of commercially available gherkins (Ajax F1-Hybrid, Bayer Company) and ten plants for each treatment were maintained. Frequent watering was done to maintain sufficient soil moisture and monitored for any emerging symptoms. Percentage of disease index was calculated after 25, 45, 65 and 85 days of planting by using the following formula[12].

$$PDI = \sum \frac{1A+2B+3C+4D+5E}{NXM}$$

Where, A, B, C, D and E are the number of leaves belongs to corresponding grades of disease severity. The leaves were rated according to the scale 1, 2, 3, 4 and 5 respectively[13]. N is total number of leaves observed and M is maximum grade of disease. For estimating different grade of disease, infected leaves on the plants were classified into five categories as follows;

0 - No symptoms on the plant;

1 - from 0 up to 25% infected leaf on the plant;

2 - more than 25% up to 50% infected leaf on the plant;

3 - more than 50% up to 75% infected leaf on the plant;

4 - more than 75% up to 100% infected leaf on the plant.

Statistical analysis

All the experimental results were statistically analyzed by using SPSS16.0 version. The data was calculated by using analyses of variance (ANOVA) and Post Hoc Tukey test ($P \leq 0.05$) and for non volatile toxicity, analyses of variance (ANOVA) Duncan's multiple range test (DMRT) using Graphpad prism 5.0 version software.

RESULTS AND DISCUSSION

Isolation and identification of pathogen and rhizospheric mycoflora

The causal agent of the outbreaks in surveyed area on gherkin was identified as *Didymella bryoniae* based on original host species, colony morphology, original host, microscopic observation of reproductive structures, and disease symptoms. Molecular characterization was conducted to confirm our identification[3]. Similarly, the ITS region of the nuclear ribosomal DNA of bioagents were amplified with the primers ITS1 and ITS4. Ambiguous regions at both ends were excluded from the analyses. The sequences of each isolates were compared with the NCBI database GenBank using the blastn algorithm. Blast results of the ITS sequences of the four isolates that produced a PCR product (deposited in GenBank as accession numbers MF503607-MF503610) confirmed that the strains were *Aspergillus terreus* ATKGM1 (MF503607), *Penicillium purpurogenum-1* PPDMK1 (MF503608), *Penicillium purpurogenum-2* PPDMK2 (MF503609) and *Trichoderma harzianum* THRHG1 (MF503610) with 100 % similarity for each of the isolate.

Effect of non-volatile (culture filtrate) compounds of bioagents on the growth of *D. bryoniae*

The effect of non-volatile compounds of bioagents on the growth of *D. bryoniae* revealed that, culture filtrate of all the four bioagents were effective in suppressing growth of the pathogen (Table 2; Figures 1 and 5). However, within treatments, *T. harzinium* showed significantly promising bioagent in

suppressing the growth of pathogen at all concentrations which was on par with commercial fungicides Kavach (0.025%) and Allite (0.025%). *A. terreus*, *P. purpurogenum-1* and *P. purpurogenum-2* were effective at 20% concentration with 28.5%, 26.0% and 23.5% of growth inhibition of pathogen respectively. At 15% concentration moderate inhibitory effect was observed in *A. terreus* (26.8%), *P. purpurogenum-1* (16.8%) and *P. purpurogenum-2* (20%). Whereas at 10% concentration little growth of pathogen was observed in all the treatments, but in 5% concentration, lowest inhibition was observed in all the treatments except *T. harzianum* (Table-2) which corroborate the earlier reports[14,15,16,17].

Evaluation of fungicides by poison food technique

It was observed that all the fungicides showed significant difference in their effectiveness and significantly inhibited mycalial growth of pathogen compared to control (Table-4; Figures 2 and 6). Amongst the five tested fungicides, Indofil and Nativo proved to be the most effective with 100% growth inhibition at 0.025% compared to Allite which showed 100% inhibition at 0.01% concentration. However, Kavach and Allite were least effective against the pathogen even at higher concentration compared to other fungicides (Table-4). It was also observed that Sectin was not effective against pathogen at any concentration. Earlier reports suggests that, effect of fungicides individually and in combination against *D. bryoniae* was effective[6,18,19], however, indiscriminate use resulted in evolution of fungicide insensitive strains resulting search for alternative methods.

Effects of bioagents on plant growth and disease incidence in green house experiment

In vivo studies against *D. bryoniae* revealed that application of bioagents extensively reduced the disease incidence and increased the growth of plant by promoting shoot length and number of leaf when compared to control. ATKGM1 isolate amended pot showed minimum percentage of disease incidence with 43.8% after 66 days of plant growth compared to other bioagents. In PPDMK1 isolate amended pot, moderate percentage of disease incidence was observed with 44.6% after 66 days of plant growth. Whereas in THRHG1 isolate amended pot, maximum percentage of disease incidence was observed and was found to be least effective against *Didymella bryoniae* (Table 3; Figure 5) which correlates earlier results[20,21].

The growth of the plant varied in different bioagents amended pots. The 66 days old plants of PPDMK1 amended pot showed highest shoot length of 62.0±8.3cm, ATKGM1 amended pot showed 54.2±7.9cm which was much higher than -ve control (18.16±0.7cm) and +ve control (9.2±3.3cm). On the other hand number of leaves were also moderately increased in PPDMK1 amended pot with 11.1±1.8 and highest in ATKGM1 with 13.5±1.0 and lowest inTHRHG1 with 10.8±1.3 compare with overall control (10.8±0.4), -ve control (6.2±1.4) and +ve control (10±0.4). The results clearly show that both isolates significantly decreases the disease incidence and increases the shoot length and number of leaf compared to control and were also effective against *Didymella bryoniae* which is in agreement with previous reports, where fresh and dry weight was increased by applying *A. niger* and *T. harizanium* and also controlled wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* under green house[22]. Furthermore wilt of tomato and brinjal crop were also effectively controlled by not only *Aspergillus* sp. but also *Penicillum* and *Trichoderma* sp.[15], *Sclerotinia* stem rot of oilseed rape was controlled by *Aspergillus* sp. and strongly recommended utilization of *Aspergillus* sp. as biocontrol agent due to its parasitic potential in decreasing the formation of Apothecia in field condition[23], *Rhizotonia solani* infection was decreased by application of *Aspergillus* sp. in egg plants[24]. The seeds coated with *A. terreus, Pencillium* sp., *T. harzianum* showed better results in controlling many soil borne pathogen in field condition[25].

Table 1: Percentage inhibition of mycelial growth of *D. bryoniae* by cultrate filtrate of native bio-agents at different concentration in poisoned food technique

SL.NO	Concentration of Bioagents	Colony diameter	% of inhibition
1	THRHG1- 5%	4.85 ± 0.31 *	18.4
2	THRHG1-10%	3.83 ± 0.40 **	35.6
3	THRHG1-15%	3.76 ± 0.30 ***	36.8
4	THRHG1-20%	3.38 ± 0.92 **	43.1
5	ATKGM1-5%	4.66 ± 0.08 *	21.6
6	ATKGM1-10%	4.55 ± 0.10 **	23.5
7	ATKGM1-15 %	4.35 ± 0.20 ***	26.8
8	ATKGM1-20%	4.25 ± 0.24 ***	28.5
9	PPDMK1 -5%	5.75 ± 0.22 **	3.36
10	PPDMK1-10%	5.01 ± 0.16 **	15.7
11	PPDMK1-15%	4.91 ± 0.52 ***	16.8
12	PPDMK1-20%	4.40 ± 0.02 ***	26.0
13	PPDMK2-5%	5.60 ± 0.25 ***	5.8
14	PPDMK2-10%	5.00 ± 0.32 ***	15.9
15	PPDMK2-15%	4.76 ± 0.43 ***	20.0
16	PPDMK2-20%	4.60 ± 0.05 ***	23.5
17	CNT	5.95 ± 0.57 *	0

mean of 3 replicates. The data were analysed using Post Hoc Tukey test.P≤ 0.05. ***shows signigficance level
CNT-Control , THRHG1-*Trichoderma harzianum1*, ATKGM1-*Aspergillus terreus1*, PPDMK1-*Penicillium purpurogenum 1* , PPDMK 2- *Penicillium purpurogenum 2*

Table 2: Percentage inhibition of mycelia growth of *D. bryoniae* by commercial fungicides at different concentration

SL.NO	Concentration of Fungicides in %	Colony diameter in mean	% of inhibition
1	CNT	5.95 ± 0.5 *	0
2	S-0.025	2.70 ± 0.32 ***	54.62
3	S-0.05	2.41 ± 1.23 ***	59.4
4	S-0.075	1.65 ± 0.93 **	72.26
5	S-0.1	0.68 ± 0.68 **	88.57
6	K-0.025	3.38 ± 0.48 **	43.19
7	K-0.05	2.70 ± 0.11 ***	54.45
8	K-0.075	2.66 ± 0.15 ***	55.29
9	K-0.1	2.61 ± 0.11 ***	56.13
10	A-0.025	3.55 ± 0.05 *	40.33
11	A-0.05	3.23 ± 0.34 **	45.37
12	A-0.075	2.71 ± 0.17 ***	65.54
13	A-0.01	0.00	100
14	I-0.025	0.00	100
15	I-0.05	0.00	100
16	I-0.075	0.00	100
17	I-0.01	0.00	100
18	N-0.025	0.00	100
19	N-0.05	0.00	100
20	N-0.075	0.00	100
21	N-0.01	0.00	100

mean of 3 replicates. The data were analysed using Post Hoc Tukey test.P≤ 0.05. ***shows signigficance level
CNT-Control, S-Sectin, K- Kavach, A-Alitte, I-Indofil, N-Nativo

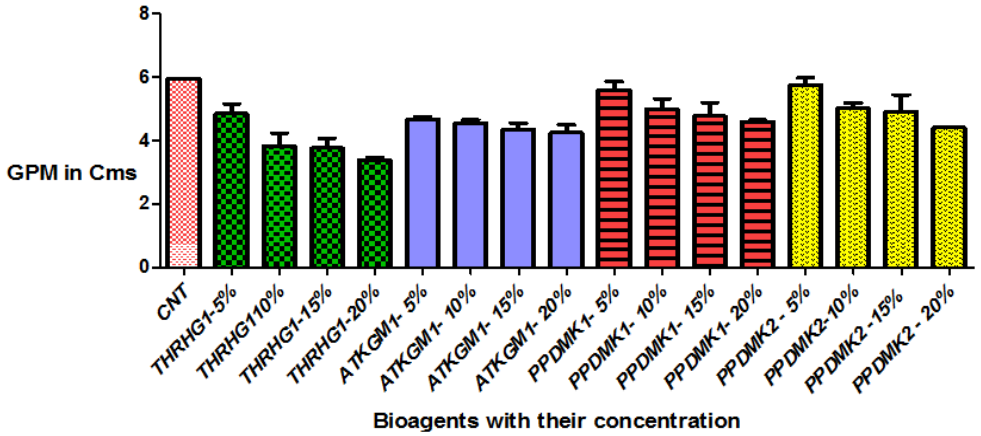
Table 3: Effects of bioagents on plant growth and disease incidence in green house experiment

TREATMENTS		PDI			MSL-MEAN SHOOT LENGTH IN CMS.			MLN-MEAN LEAVES NUMBER		
↓	*Plant Growth in days→*	25	40	66	25	40	66	25	40	66
1	OAC	20	20	20	24.1±1.6	41.8±.0	65.5±3.9	3.6±1.6	10.1±0.3	10.8±0.4
2	-VE CNT	20	50	96.5	18.16±0.7	28.5±1.8	30.9±6.3	3±0.2	7.8±0.5	6.2±1.4
3	THRHG1	20	66	74.6	17.4±1.2	22.5±1.7	27.3±1.3	3.0±0	6.1±0.7	10.8±1.3
4	ATKGM1	20	32	43.8	10.51±1.9	33.7±3.6	54.2±7.9	2.3±0.3	6.3±0.4	13.5±1.0
5	PPDMK1	20	40	44.6	13.4±0.7	29.6±3.8	62.0±8.3	1.8±0.2	5.4±0.5	11.1±1.8
6	+VE CNT	20	24.8	34.4	9.2±3.3	19.8±1.5	34.3±4.6	1.7±0.1	4.4±0.1	10±0.4

OAC- Over all control, THRHG1-*Trichoderma harzianum1*, ATKGM1-*Aspergillus terreus1*, PPDMK1-*Penicillium purpurogenum 1* , PPDMK 2- *Penicillium purpurogenum 2*, *-VE CNT*- Negative control, *+VE CNT*- Positive contol.

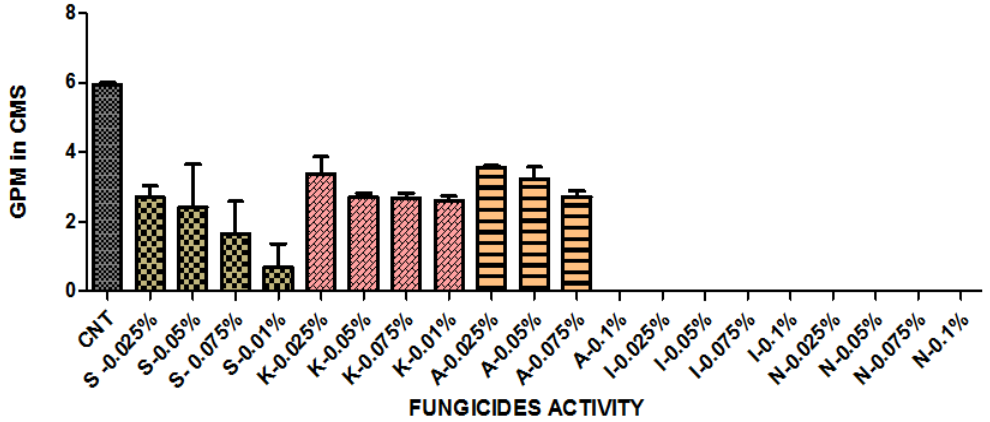
Number of leaves and shoot length were mean of 10 plants from each treatment.

Fig. 1: Percentage inhibition of mycelial growth of *D. bryoniae* by cultrate filtrate of native bio-agents at different concentration



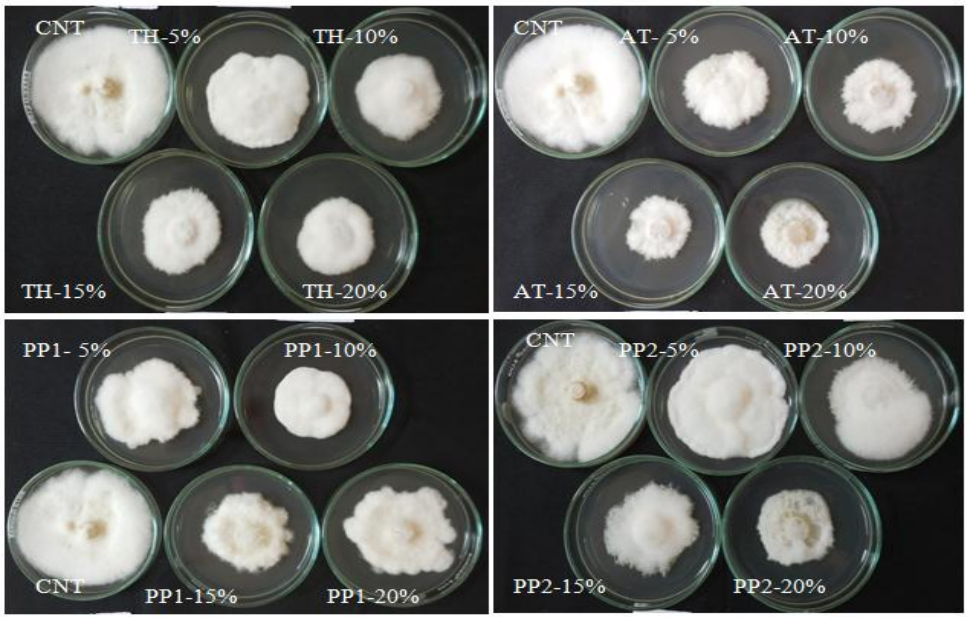
GPM-growth percentage of pathogen mycelia. CNT-Control plate , THRHG1-*Trichoderma harzianum1*, ATKGM1-*Aspergillus terreus1*, PPDMK1-*Penicillium purpurogenum* , PPDMK 2- *Penicillium purpurogenum*

Fig. 2: Percentage inhibition of mycelia growth of *D. bryoniae* by commercial fungicides at different concentration



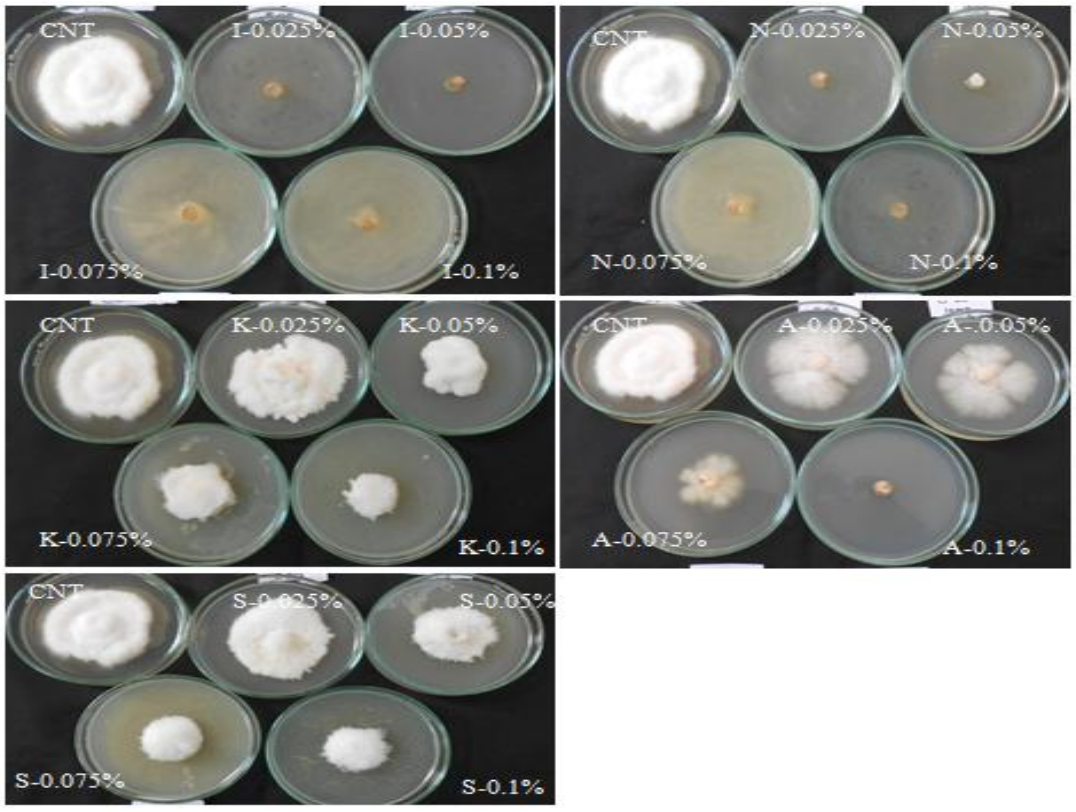
GPM-growth percentage of pathogen mycelia. CNT-Control plate, S-Sectin, K- Kavach, A-Alitte, I-Indofil, N-Nativo

Fig. 3: Effect of non-volatile compounds of bioagents at different concentration against *D. bryoniae* poisoned food technique



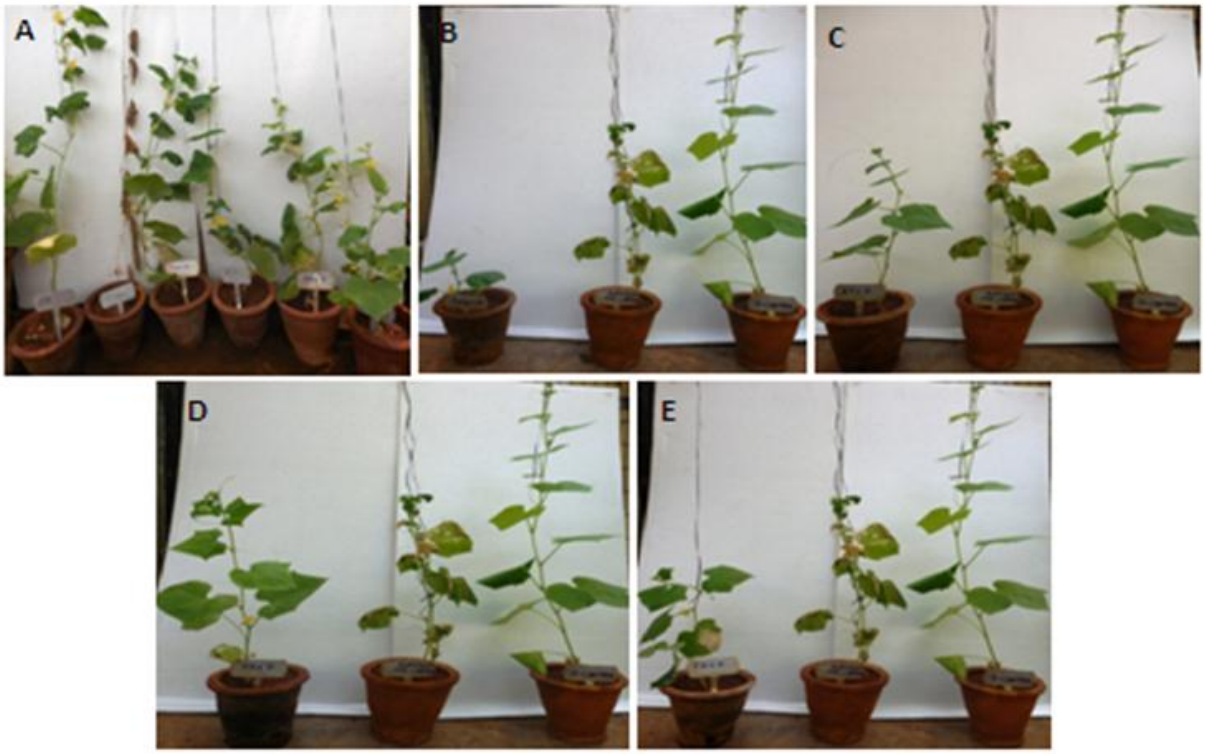
CNT- Control plate, TH-*Trichoderma harzianum*, AT-*Aspergillus terreus*, PP- *Penicillium purpurogenum*

Fig. 4: Effect of fungicides at different concentration against *D. bryoniae* in poison food technique



CNT-Control plate, S-Sectin, K- Kavach, A-Alitte, I-Indofil, N-Nativo

Fig. 5: (A) Effect of different treatments on growth and infection of Gherkin plants; (B) Fungicide applied uninfected plant compares with –ve and overall control; (C) ATKGM1 isolate applied uninfected plant compares with –ve and overall control; (D) PPDMK1 isolate applied uninfected plant compares with –ve and overall control; (E) THRHG1 isolate applied infected plant compares with –ve and overall control.



CONCLUSION

The current study proved that both *A. terreus* and *P. purpurogenum* are efficient for manage the *Didymella bryoniae* both *in vitro* and *in vivo* conditions. However, in *in vitro* condition *T. harzianum* showed good result but did not show promising results in pot experiments. Each isolates has individual prospective, not only in reducing the disease incidence but also in promoting the growth of plant by increasing shoot length and leaves number. Nevertheless, further exploration is necessary to comprehend the definite description between isolates and pathogen and their stability in field condition along with exploitation of secondary metabolites which can able to substitute for hazardous chemical fungicides which can overcome pollution along with developing an environmental friendly and economically reasonable bioagents for managing gummy stem blight disease of gherkin.

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