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Biological Control of Fungal Leafy Vegetable Diseases through Antagonistic Fungi

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Abstract

In this study, *in vitro* potential of *Trichoderma viride* was evaluated against ten fungal pathogens of leafy vegetables by dual culture technique, slide culture method and well diffusion method. Simultaneously the effect of physical parameters such as pH and temperature on *T. viride* mycelial growth was also evaluated. *T. viride* had marked significant inhibitory effect on mycelial growth of ten targeted fungal pathogens in dual culture method and slide culture method with respect to their control. Maximum percent inhibition and coiling structure were recorded in *F. moniliforme* and *S. verruculoscum.* On the other hand, in well diffusion method, *F. roesum* and *H. sativum* were found to be most susceptible to *T. viride.* Effect of pH and temperature revealed that *T. viride* had antagonistic activities under a wide range of pH and temperature.

Key Words: Biological control, *Trichoderma viride*, Leafy vegetables, Antagonistic effect

Introduction

India has achieved self sufficient and good degree of stability of vegetable crop production. Vegetables being more succulent and rich in nutrients are prone to variety of diseases right from sowing till marketing, thereby increasing in yield losses during pre and post production periods. Leafy vegetable plants are mostly suffering from the diseases caused by different microorganism such as insect, nematodes, fungi, phytoplasma, bacteria and viruses which leads to loss in quality and quantity of plant and plant parts day by day. Leafy vegetables are prone to several fungal diseases most commonly causing leaf spot and wilting. Due to these diseases annually billions of rupees loss occurs throughout the country, though 74% of Indian population is engaged in agriculture. To control these diseases, the pesticide compounds being widely used throughout the world which on contrary increasing the agricultural production with increasing pesticide concentration. But the future role of pesticide used in agriculture indicates increasing threatened by several factors like development of resistance in pathogens, accumulation of toxic compounds and loss in food safety. However there are series of problems against the effective use of chemicals in areas, where the fungi and bacteria had developed resistance [2].

The biological control agents have enormous antimicrobial potential. They are effective in treatment of infectious diseases, simultaneously mitigating many of the side effects which are associated with pesticides. So there is growing realization in the people that biological control can be successfully exploited as an agricultural method for soil borne pathogens [15]. Biological control of plant pathogen by microorganism has been considered more natural and environmentally acceptable alternative to the existing chemical methods [1]. Biological control has been developed as an alternative to synthetic fungicide treatment and considerable success had been achieved upon utilizing antagonistic microorganism to control both pre harvested and post harvested diseases [7].

Material and Methods

Isolates

Trichoderma viride was isolated from the soil by serial dilution technique. Therefore, 1 g of soil was dissolved in 10 ml of sterile double distilled water and mixed well to get 1:10 dilution. From this dilution, 1 ml was mixed in a 9 ml of sterile double distilled water and mixed well, this was 1:100. In the same manner 1:1000 dilutions was prepared. Out of these various soil dilution; 1 ml was inoculated in fresh PDA plates and incubated for 28 ± 2 ^{o}C for 7 days. After incubation period the growth of different fungal pathogen was observed microscopically and *T. viride* was identified by using soil manual [8].

Pathogens

Ten plant pathogenic fungi were isolated from eight leafy vegetables such as *Anethum graveolens* L., *Brassica oleracea* L., *Carthamus tinctorius* L., *Colocasia esculanta* L., *Coriandrum satvium* L., *Rumex vesicarius* L., *Spinacia oleracia* L. and *Trigonella foenum-graecum* L. The Infected leafy vegetables plant parts were separately collected in sterilized polythene bags and were brought in the laboratory. Afterwards, these infected plant part from the advancing margin of lesions were cut into small pieces (2-5mm) and kept in sterile Petri plates separately. The pieces were dipped into 0.1% mercuric chloride ($HgCl_2$) solution for about one minute. These pieces were transferred to Petri plates containing sterile double distilled water to free them from the chemical trace and

saprophytic microorganisms if any. After washing, 2-4 pieces were placed at equal distance on a fresh solidified Potato Dextrose Agar (PDA) medium plate in aseptic condition with the help of sterile forceps. Then the plates were incubated at 25 ± 3 °C for 3-7 days and examined daily for the growth occurrence of the fungus. After the incubation period, the fungal cultures were examined under microscope and correctly identified by using standard literature.

Among the different isolated plant pathogenic fungi, only 10 fungi namely, *Alternaria brassicae*, *Alternaria carthami, Alternaria humicola, Collectotrichum lindemuthianum, Fusarium moniliforme, Fusarium oxysporum, Fusarium roseum, Helminthosporium sativum, Pullularia pullulans, Stemphylium verruculosum* were selected as target pathogens. These selected pathogens were purified by sub culturing them in fresh PDA plates.

1) Dual culture method

Trichoderma viride was evaluated against ten phytopathogenic fungi of leafy vegetables by dual culture method [12]. A mycelial disc of 4 mm diameter from the margin of 7 days old culture were obtained from both, *Trichoderma* species and targeted fungal pathogen of leafy vegetables and were dually placed on fresh PDA medium plates opposite to each other and at equal distance from the periphery. In control plates, a sterile disc Whatman No. 1 filter paper of 4 mm diameter, was placed at opposite side of targeted fungal pathogens in complete aseptic condition. The experiment was carried out in triplicates and targeted fungal pathogen separately. Then inoculated plates were incubated at 25 ± 1°C until the end of incubation period of 7 days after the inoculation. After complete incubation period, the radial growth of each targeted fungal pathogen and radius of inhibitory zone was measured in mm. Percent inhibition of mycelial growth of each tested pathogen in relation to growth of control were calculated using formula given by [19].

$$I = \frac{100\,(C-T)}{C}$$

Where I = Inhibition of mycelial growth.
C = Mycelial growth in control
T = Mycelial growth in treated.

2) Slide culture method

Assessment of antifungal activity of *T. viride* was evaluated against ten phytopathogenic fungi of leafy vegetables by slide culture method [16]. A clean slide was placed on a Z-shaped glass rod in 90 mm Petri dish and autoclaved. Then small amount of molten PDA medium was poured and evenly spread over the slide to make a thin agar film with one end of the slide kept free to facilitate handling. Thereafter, a disc of 4 mm diameter of 5-7 days old culture of both *Trichoderma spp.* and targeted fungal pathogen were placed on the slide 1 cm apart from each other in aseptic condition. A few ml of sterile distilled water was added to Petri dish to prevent drying of the slide and incubated at 28 °C for 5 days. At the end of incubation period, region where the hypae of *T. viride* met the hypae of targeted pathogens were observed under light microscope for the presence of coiling structure and cell wall disintegration. The frequencies of coiling structure and cell wall disintegration were estimated by counting the coiled area with disintegrated cell wall under light microscope in three different fields.

3) Well diffusion method

The antagonistic activity of *T. viride* against ten fungal pathogens of leafy vegetables was studied by well diffusion method [16]. 100 ml potato dextrose broth was inoculated with three mycelial disc of 4 mm diameter obtained from the 5-7 days old culture of *T. viride.* The inoculated medium was incubated at 28 °C on orbital shaker at 80 rotations per minute (rpm) for 12 days. At the end of incubation period, the culture filtrate was harvested by filtering through two layers of muslin cloth. The obtained culture filtrate was concentrated by freeze drying.

The fresh PDA plates were prepared which had four wells of 10 mm diameter prepared with the help of cork borer situated at 1 cm away from the periphery and equidistance from each other. Then the culture filtrate 0.1 ml was placed in each well of the PDA plates and immediately afterwards, a 4 mm diameter mycelial disc of targeted pathogen was placed at the center of Petri plates. In control plates, autoclaved culture filtrate was used instead of normally prepared *T. viride* culture filtrate. Then all inoculated plates were incubated at 28 °C for 12 days. Observation were made for measurement of radial growth of targeted pathogen and percent inhibition of average mycelial growth was calculated in relation to growth in control by using following formula [19],

$$I = \frac{100\,(C-T)}{C}$$

Where I = Inhibition of mycelial growth.
C = Mycelial growth in control
T = Mycelial growth in treated.

Effect of physical factor on fungal biocontrol agent

The effect of pH and temperature on the fungal biocontrol agent; *T. viride* was recorded. The different PDA mediums were prepared by adjusting their pH to 6, 7, 8 using 0.1 N HCl and NaOH before autoclaving. After complete sterilization, medium was poured into sterile Petri plates in aseptic condition and allowed to solidify. Then 4 mm diameter mycelial disc from the margin of 5-7 days old culture of *T. viride* was inoculated centrally and all the plates were incubated at varied degree of temperature like 25 ± 1 °C, 30± 1 °C and 35 ± 1 °C respectively. After incubation period of 6-7 days, the colony diameter of *T. viride* was recorded by mean of triplicates.

Result

When all the pathogenic fungi were tested in combination with *T. viride,* among them *F. moniliforme, S. verruculosum, A, carthami* and *F. oxysporum* were found to be most susceptible and revealed highest percent of inhibition of mycelial growth i.e. 40.78%, 40.32%, 35%, and 34.52% respectively. On contrary, *H. sativum* and *F. roseum* were the most resistant and revealed lowest percent inhibition as 18.46% and 17.14% of mycelial growth respectively. And all the tested fungal pathogen in combination with *T. viride* showed inhibitory zone in the range of 0 to 4 mm in diameter (Table 1).

On the other hand ten fungal pathogens of leafy vegetables were also tested against *T. viride* by slide culture method, the pathogen *F. moniliforme* and *S. verruculosum*

were most sensitive and showed 58 and 57 number of coiling structure with 18 and 16 number of disintegrated mycelium while, *F. roesum* and *H. sativum* were more resistant and showed 22 and 20 number of coiling structure with 6 and 7 number of disintegrated mycelium. On the other hand remaining six fungal pathogens showed 29-54 number of coiling structure with 9-16 number of disintegrated mycelium (Table 2).

When all ten fungal pathogen of leafy vegetables tested separately in combination with *T. viride* by well diffusion method, the pathogen *F. roseum, H. sativum* and *F. oxysporum* were more susceptible and showed 77.33%, 77.01% and 73.75% inhibition of mycelial growth respectively. Whereas *F. moniliforme, A. brassicae* and *C. lindemuthanium* were most resistant and revealed 66.10%, 66% and 62.96% inhibition of mycelial growth (Table. 3).

Effect of pH

Tested biocontrol agent *T. viride* was exposed to different pH such as 6, 7 and 8 respectively and the influence of pH on the mycelium growth was recorded (Table. 4). At pH 7, *T. viride.* showed highest mycelial growth as 32mm. While at pH 6 and pH 8 the growth rate was significant that is 30mm and 29mm respectively. It indicates that pH 7 which neutral is effective for *T. viride* maximum mycelial growth after 7 days of incubation period.

Effect of temperature

In the same manner, *T. viride* was exposed to different temperature such as 25 ^{o}C, 30 ^{o}C and 35 ^{o}C (Table. 4). The influence of the varied temperature on the mycelial growth rate was tabulated. Basically at temperature 30 ^{o}C, *T. viride* revealed 36 mm mycelial growth. Whereas, lowest growth was occurred 30 mm at 25 ^{o}C temperature while at 35 ^{o}C, significant growth was recorded as 32 mm. Therefore, the suitable temperature for *T. viride* was 30 ^{o}C, which had showed active growth rate.

Table 1. Antagonistic effect of *T. viride* against on mycelial growth of ten leafy vegetable pathogens by dual culture method

Pathogen	Mycelial growth in control (mm)	Growth rate in *T.viride* + pathogen combination (mm)	% inhibition of Mycelial growth over control	Radius of inhibitory zone (mm)
A. brassicae	76	52	31.57 ± 1.63	2 ± 0.66
A. carthami	60	39	35 ± 0.47	1 ± 0.47
A. humicola	64	44	31.25 ± 1.24	2 ± 0.94
C. lindemuthianum	70	48	31.42 ± 0.81	2 ± 0.64
F. moniliforme	76	45	40.78 ± 1.41	0 ± 0.00
F. oxysporum	84	55	34.25 ± 2.05	1 ± 0.57
F. roseum	70	58	17.14 ± 1.70	4 ± 0.47
H. sativum	65	53	18.46 ± 1.37	4 ± 0.94
P. pullulans	66	49	25.75 ± 1.24	3 ± 0.81
S. verruculosum	62	37	40.32 ± 1.91	0 ± 0.00
C.V.			4.84	45.97

Values expressed in mean ± S.E.M. of triplicates

Table 2. Antagonistic effect of *T. viride* on mycelial growth of ten pathogenic fungi leafy vegetable by slide culture method.

Pathogen	Number of coiling structure	Number of disintegrated mycelia
A. brassicae	49	13
A. carthami	54	16
A. humicola	50	13
C. lindemuthianum	50	14
F. moniliforme	58	18
F. oxysporum	29	9
F. roseum	22	6
H. sativum	20	7
P. pullulans	29	10
S. verruculosum	57	16
Mean	41.8	12.2

Table 3. Antagonistic effect of *T. viride* against ten pathogenic fungi of leafy vegetable by well diffusion method.

Pathogen	Mycelial growth in control (mm)	Mycelial growth in *T. viride* + pathogen combination(mm)	% inhibition of mycelial growth over control
A. brassicae	50	17	66 ± 0.94
A. carthami	53	16	69.81 ± 0.41
A. humicola	55	15	72.72 ± 1.33
C. lindemuthianum	54	20	62.96 ± 1.41
F. moniliforme	59	20	66.10 ± 1.63
F. oxysporum	80	21	73.75 ± 1.70

F. roseum	75	17	77.33 ± 0.94
H. sativum	87	20	77.01 ± 1.79
P. pullulans	59	18	69.49 ± 0.81
S. verruculosum	50	16	68 ± 1.24
C.V.			11.55

Values expressed in mean ± S.E.M. of triplicates.

Table 4. Effect of physical parameter on the growth rate of *T. viride*.

Parameter	Range	Growth rate
		T. viride
pH	6	30
	7	32
	8	29
Temperature	25 °C	30
	30 °C	36
	35 °C	32

* All values expressed in mean of three replicates.

Discussion

Research in agricultural has been directed to fungi as a biocontrol agent against both fungi and insect. Most of fungal antagonistic have been used because of their high antifungal properties [9]. Hence biological control has been developed as an alternative to synthetic fungicide treatment and considerable success had been achieved upon utilizing antagonistic microbes to control both pre harvested and post harvested diseases [7].

Over 75 years ago [20] demonstrated that antagonistic nature of fungal species from the genus, *Trichoderma.* In the present study, the result of dual culture method revealed the rapid colonization of the medium by *Trichoderma viride* with 34.12% overall antagonistic activity. Similar findings were reported by several workers [13 and 11]. *T. harzianum* showed greatest antagonistic activity against *G. microchlamydosporum, C. gloeosporioides* and *C. microchlamydosporum* [16]. The results of the present study were similar with previously reported results of [5] in which *T. viride* was more effective than *T. hamatum* in antagonistic assay. The antagonistic activity was due to the secretion of harmful extracellular compounds released by *Trichoderma* sp. The similar phenomenon was described by number of workers [17,3 and 4] according to them the degree of effectiveness varies according to the nature, quality and quantity of antibiotics and inhibitory substances secreted by the antagonists.

The antagonistic effect of three *Trichoderma* sp. was also studied by using slide culture method in which specially *T. viride* revealed high chemical antagonism by producing cell wall degrading enzymes which causes disintegration of cell wall of targeted pathogens with mycoparasitism activity by creating coiling structure around the mycelium of targeted pathogens. Among the tested pathogens averagely *F. moniliforme* was averagely most sensitive and showed 56 number of coiling structure with 16 number of disintegrated mycelium. While *H. sativum* was most resistant one which revealed 21 number of coiled structure with only 7 number of disintegrated mycelium. Similar findings were recorded [16], 72 coiling structure with 40 number of disintegrated mycelium in *G. microchlymadosporum* by *Trichoderma* sp. Coil formation is a common form of mycoparasitism and leads to death of parasitized fungi [18].

Well diffusion method was also selected to study antifungal activity of *T. viride* against ten fungal pathogens of leafy vegetables. *T. viride* was highly effective and revealed averagely 70.31% inhibitory activity. Among tested pathogens, *F. roseum* was most sensitive with 72.44% and *C. lindemuthianum* was most resistant with 59.25% against all *Trichoderma species. In-vitro* test of antagonistic activity of culture filtrates from *T. harzianum* Rifai and *T. pseudo-koningii* Rifai strains against post harvested pathogens of some fruits [14].

The effect of two key parameter, pH and temperature on the growth rate of *T. viride,* was differed. At pH 7, *T. viride* showed maximum mycelial growth as 32 mm, while at temperature 30 °C the growth rate was significant as 36mm. It indicates that pH 7 and temperature 30 °C are favorable physical parameter to *T. viride* for active growth and other inhibitory mode of action such as mycoparasitism, competition and antibiosis. *Trichoderma* strains are highly active and effective at specific pH and optimum temperature although they are mesophilic[10]. Hajieghrari et al. [6] was studied the effect of pH and temperature on the growth rate of six isolates of *Trichoderma species.*

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