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Bioagents *Trichoderma* and *Pseudomonas* Shows Promising Results against Isolates of Sugarcane Pathogen *Colletotrichum falcatum* from Punjab Region

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ABSTRACT

Background: Red rot causing fungus *Colletotrichum falcatum* is most destructive and severe pathogen of sugarcane. Genetic makeup of this pathogen *C. falcatum* changes continuously and management of this disease is difficult by fungicides. Biological control offers fair opportunity to manage the yield loss caused by this pathogen. Aim of this research was to study the pathogenic behavior of *Colletotrichum falcatum* isolates in Punjab region and also to evaluate the potential of the biocontrol agents against the *Colletotrichum falcatum.*

Methods: Pathogen isolates were collected from sugarcane mill areas of Punjab during survey and their pathogenic behaviour on fourteen sugarcane differentials was also studied against *C. falcatum*. Further efficacy of biocontrol agents were studied against red rot pathogen in both *in vitro* and *in vivo* conditions

Result: CF02 pathotype showed higher virulence on sugarcane differentials and pathotype CF01 showed less virulence. Four sugarcane differentials, Baragua, BO91, COS8436, SES594 were found resistant to all the pathotypes or isolates. Studies revealed that all the antagonists inhibited or suppressed the mycelial growth of *Colletotrichum falcatum* out of that *Pseudomonas flourescens* found to be more efficient by showing the inhibition control of 68.18% *in vitro* conditions. *Trichoderma viride* was found to be highly effective in vivo conditions, by controlling the red rot disease up to 32.2%. Overall present study shows management of plant pathogens through biological control is a promising and sustainable measure for necrotrophic pathogen like *C. falcatum* in current scenario.

Key words: Antagonists, Biocontrol, *Colletotrichum falcatum*, Differentials, Red rot, Sugarcane.

INTRODUCTION

Sugarcane (*Saccharum officinarum*) holds a prominent position as a cash crop in India. Area under sugarcane in India is 4.90 million ha with productivity of 70.7 t/ha, sugarcane production 350 million tonnes and sugar production about 27.9 million tones (Anonymous, 2017a). In Punjab state alone, the area under sugarcane is 92 thousand ha with cane yield 73.3 t/ha, sugarcane production 6.6 million tones and sugar production 0.5 million tones (Anonymous, 2017b). However, some of the abiotic and biotic factors are responsible for its low yield, one of the major causes of concern is the diseases. Estimated loss due to fungal diseases in India is about 18 to 31 percentage. Crop is reported to be affected more than hundreds of disease from the stage of planting but the red rot, devastating fungal disease which causing the major threat to sugarcane crop. Red rot is widely distributed in almost sugarcane growing countries in the world. It is causing loss in both cane yield and sugar recovery of about 29.07% and 30.8%, respectively (Husssnain and Afghan 2006).

Red rot was first reported by Went from Java (now in Indonesia) in 1893. First scientific observation of red rot disease was made by Barber (1901) at Red Mauritius in the Godavari delta Andhra Pradesh (India). Subsequently many outbreaks have reported in Jammu and Kashmir in 1922 and in Pusa (Bihar) in 1932. The disease attract special attention during the year 1938-1940. Then it appeared in an epidemic form in Northern Bihar and Western Uttar Pradesh resulting in failure of most popular variety CO213 (Chona and Padwick, 1942. The disease again appeared in epidemic form in the year 1946-1947 in several districts of Uttar Pradesh resulting in failure of the popular variety CO312 (Mathur 1946).Outbreak of this disease has also been responsible for failure of variety CO1148, COJ64 in Punjab, Haryana and Western Uttar Pradesh (Satyavir, 1984).

Red rot is caused by the *Colletotrichum falcatum* (Sexual stage *Glomerella tucumanensis*) is a facultative saprophyte. The colonies of the fungus are dirty, white or dark brown to black. The hyphae are slender, septate, branched, brown. The setae are dark brown to black. Condiophores are short, erect, simple, hyaline, non-septate.

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Conidia are hyaline but pinkish in mass, unicellular, thin walled, falcate to lanceolate, oil globule in Centre. Disease expression vary under prevailing environmental conditions (Satyavir; 2003). It is very difficult to recognize this red rot disease during initial stage (Nithya *et al*; 2012). Entire plant is infected by the pathogen including stalk, leaves, buds and also the roots. Characteristic symptoms of the disease is observed in field during rainy season and post monsoon period. The external symptoms appear after 16-21 days after infection and drying of entire canes takes place after 10 days' time. The most damaging stage of this disease is when the pathogen attacks the stalk region. When the affected canes split opened, the inner region is reddish in color with intermittent white tinges and exhibit alcohol odour. The type of symptoms varies based on the factors like age of the stalk, time of infection and susceptibility of the cane genotype (Duttamajumder, 2002, 2008).

Large quantity of water is consumed by the sugarcane crop, so the moisture level plays a major role in the disease development. Rainy season and the temperature level of 25-30 degree Celsius also favour the development of the disease. High atmospheric humidity of 90% is also responsible for disease occurrence. Pathogen is set borne. perpetuation of the fungus is through infected canes, crop debris and disease debris and also in soil it survives in the form of mycelium, appressoria, setae, chlamydospores, conidia. These resting structures can survive in the soil for longer period of time. Infected planting materials are the source of primary inoculum. The secondary transmission is during monsoon season and mediated through the irrigation, rain, rain splash in north western part of country the environment conditions are more favourable for disease development so that disease is more severe in the north western parts of India (Singh *et al*; 1988).

Biological control considered as an effective tool and a main component of the integrated disease management. Combination of fungal and bacterial antagonists were found to be more effective to the crop. Red rot is biologically control through *Trichoderma harzianum (*Kapat *et al*., 1998*)*, *Trichoderma viride* and the *Pseudomonas fluorescens*, they have the ability to protect the crop from soil borne inoculum. Efficacy is due to the chitinase enzyme which is produced by them. The ech42 gene which present in *Trichoderma spp* is effective for control of the red rot disease and salicylic acid will boost the protection level and also help to induce systemic resistance. *Trichoderma* is used as biopesticides, biofertilizers and also for soil amendments too. *Trichoderma harzianum* is the potent bioagents which is used against the red rot of sugarcane (Singh *et al*;1994, Singh *et al*., 2004, Meena *et al*., 2016). Previous studies also showed inhibitory effect of *Pseudomonas* and *Trichoderma* isolates against red rot pathogen under *in vitro* and *in vivo* conditions in sugarcane (Nallathambi *et al*; 2000) Present work investigate red rot incidence in Punjab India and collection of pathogen from different area and testing antagonistic activity against biocontrol agent *viz. Trichoderma* spp. and *Pseudomonas.*

Table 1: Isolates of *Colletotrichum falcatum* and their place of collections.

Isolates	Varieties	Origin of cultivar/state
cf-01	Co 89003	Nawanshahar mill area, Punjab
Cf-02	Co-j 85	Bhogpur mill area, Punjab

MATERIALS AND METHODS

Survey of Punjab state for estimation of red rot incidence in sugarcane

Surveys was conducted for recording the incidence of red rot in sugarcane varieties growing in the 4 sugar mills (*viz*, Bhogpur, Nawanshar,) were carried out. Doaba area is located at 30° 57′ -32° 07°N and 75° 04′ -76° -30°E at an altitude of 270-300 m.a.s. Two rounds of disease survey in sugarcane crop were carried out during September and November month in Punjab state in the year of 2021. During survey the prevalence and severity of red rot were observed. The diseased plant samples were also collected for further laboratory and field studies. Soil sample one kg/field for bioagents were also collected for further studies.

Collection and maintenance of red rot isolates

Red rot infected canes were collected from different varieties/ clones from major sugarcane growing mills areas of Punjab (Fig 1). The plants showing typical symptoms were taken for further studies. Isolates of *Colletotrichum falcatum* and their place of collection is listed here (Table 1). Isolates were maintained on oat meal agar medium with subculturing and stored for later use.

Fig 1: Red rot infected canes.

Culture and morphological variability

Mycelial disc of 5 mm diameter which is taken from the 7 days old culture were inoculated in the middle of oat meal agar petri plates. In a BOD incubator, these cultures were incubated at a temperature of 25±1°C. After 7 days of incubation, morphological and cultural traits such as colony diameter (mm), growth pattern and sporulation were noted.

Pathogenicity assay

A total of 14 sugarcane differentials, including Baragua (*Saccharum officinarum*), Kabai (*Saccharum sinense*), SES 594 (*Saccharum spontaneum*), CoS 767, Co 995, BO 91, CoC671, Co 7717, Co 997, Coj 64, Co 1148, Co 419, Co 419, Co 62399 and Co 8436, were used to study the pathogenic variability of *Collectotrichum falcatum* After 60 days of inoculation, disease data was recorded by splitting the canes longitudinally. These differentials were inoculated using the plug method (Srinivasan and Bhatt, 1961). According to Srinivasan and Bhatt (1961), observations were made on a scale of 0 to 9. The results were classified as Resistant (0-4.0), Intermediate (4.1-6.0) and Susceptible (>6.1). The research was carried out in school of agriculture at Lovely Professional University in Punjab for 2 years.

Isolation and maintenance of biocontrol agents

Biocontrol agents were isolated from the soil samples using serial dilution method which is collected during survey.*Trichoderma* selective medium (Elad and CHET, 1983, Belaidi *et al*., 2022) is used for maintenance of *Trichoderma* species and *Pseudomonas* species were maintained by using King B media. These isolates were sub cultured and kept for later use.

Efficacy test *in vitro*

Efficacy of biocontrol agents were assessed by dual culture technique on oat meal agar at 28-30^{0}C. Observations, were recorded on seventh day after inoculation (Malathi *et al*. 2008). Inhibition percentage of *C. falcatum* growth was calculated by the formula;

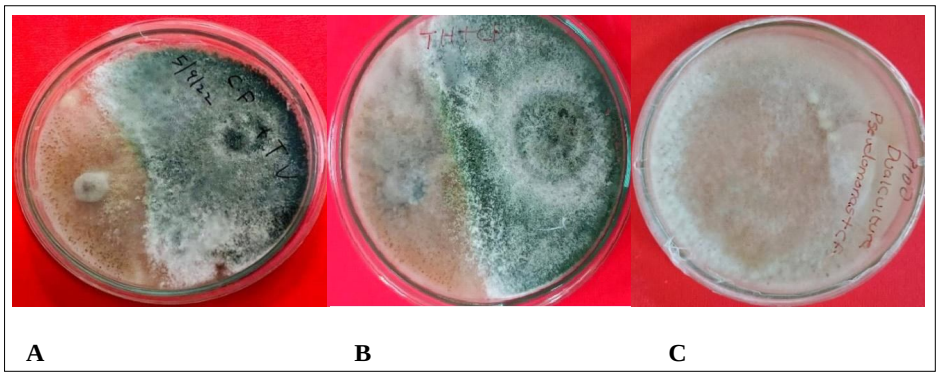
$$\frac{C - T}{C} \times 100$$

Table 2: The treatments description.

Treatments	Description
T_1	Sett treatment with Bavistin @ 0.25% (Healthy control).
T_2	Inoculation of *Colletotrichum falcatum* (10^6 conidia /ml) on sugarcane setts.
T_3	*Trichoderma harzianum* formulations (*Trichoderma* spp culture were multiplied in Farm Yard Manure was applied in soil @ 20 kg/ha) were applied in soil and sett treatment (dipping in spore suspension of 10^6 conidia / ml) at the time of planting.
T_4	*Trichoderma viridae* formulations (*Trichoderma* spp culture were multiplied in Farm Yard Manure was applied in soil @ 20 kg/ha) were applied in soil and sett treatment (dipping in spore suspension of 10^6 conidia / ml) at the time of planting.
T_5	Sett treatment with *Trichoderma harzianum*(dipping in spore suspension of 10^6 conidia / ml) which followed by the Inoculation of *Colletotrichum falcatum* (10^6 conidia /ml) on sugarcane setts.
T_6	Sett treatment with *Trichoderma viridae* (dipping in spore suspension of 10^6 conidia/ml).which is followed by the Inoculation of *Colletotrichum falcatum* (10^6 conidia /ml) on sugarcane setts. The recommended package of practices was followed during sugarcane crop growth. Germination was recorded after 40 days of planting.

Table 3: Colony Characters of isolates of *Colletotrichum falcatum.*

Isolates	Colony colour	Mycelium pattern	Sporulation	Length*1 (µm)	Breadth*1 (µm)
Cf-01	Greyish white	Fluffy	Medium	37.0	10.0
Cf-02	Greyish white	Fluffy	High	26.0	5.5



A **B** **C**

Fig 2: Dual culture (A- *Trichoderma viridae+Colletotrichum falcatum*, B-*Trichoderma harzianum+Colletotrichum falcatum,* C-*Pseudomonas fluoresens+Colletotrichum falcatum*.).

C = Radial growth of the fungus in control.
T = Radial growth in dual culture (Dubey 1998).

Efficacy test *in vivo*

Sugarcane varieties namely CoJ 85 and CoJ 88 (2 budded) was used in field trials**.** The trials were laid out with six treatments (Table 2) with a plot size of 15 m * 8 m. Efficacy of treatments was evaluated on the basis of percent germination and plant survival under the impact of soil borne inoculum of red rot recorded at different time intervals.

RESULTS AND DISCUSSION

Morphological and cultural variability

Observations showed that Cf-01 and Cf-02 isolates are having specific morphological features (Fig 2). Mycelium is of greyish white with cottony texture. Cf-01 is having less sporulation compare to Cf-02 isolates (Table 3).

Pathogenic variability

4 pathotypes/isolates did not exhibit any pathogenic behaviour which is similar to that of another pathotype/ isolate when tested on a set of 14 host differentials. The CF02 pathotype isolated from Co89003 showed the highest level of virulence among the four specified pathotypes, followed by CF08, CF03 and Cf-01 pathotypes on the set of 14 host differentials. The pathotype CF02, which was discovered to be highly virulent, induced 10 S and 4 R reactions on 14 host differentials, followed by CF08 (9 S and 5 R), CF03 (5 S and 9 R) and CF01 (4 S, 9 R and 1X) (Table 4).

The CF03 and CF01 pathotypes were discovered to be less pathogenic since they only showed 4-5 S and 1 X reactions on 14 host differentials. CF03 pathotype showed susceptible reaction on its host cultivar that is COJ 64. The pathotype Cf 02 showed more virulence on numerous differentials, but not as much on their host cultivars (Table 3). Four sugarcane differentials namely BO 91, Baragua, CoS 8436 and SES 594 were resistant to all pathotypes/isolates.

Red rot pathogen adaption is very well established to different sugarcane cultivars (Srinivasan 1962). Previous studies indicated that the pathogenic organism slowly trying to adapt a new cytoplasm and developing tolerance to host and lastly change virulence against the hosts (Viswanathan, 2010). According to research from ICAR-SBI, Coimbatore, the same *C. falcatum* pathotype which is repeatedly inoculated into incompatible host and pathogen reactions, increasing the virulence of a less virulent phenotype (Padmanaban *et al*, 1996) and additional research by the same group demonstrated that pathotypes differ in their capacity to produce hydrolytic enzymes during their host-pathogen interactions, including cellulolytic, pectinolytic and melanolytic enzymes. Pathogen virulence was also discovered to be associated with the virulence of *C. falactum* (Malathi *et al*, 2012a) Besides mutations, the possibility of a new race emerging in *C. falcatum* hybridization could not be excluded (Agnihotri 1990).

Table 4: Pathogenic behaviour of red rot isolates on sugarcane differentials.

Isolate No.	Varieties	Differentials												
		Baragua	Khakai	SES 594	CoS 767	BO 91	CoC 671	Co 7717	Co 995	CoJ 64	Co 1148	Co 419	Co 62399	Co 997	CoS 8436
Cf-01	Co89003	3.4 R	8.5 S	2.4 R	2.4 R	3.6 R	4.1 X	3.8 R	1.4 R	8.5 S	3.2 R	3.5 R	8.5 S	8.8 S	2.2 R
Cf-02	CoJ85	3.3 R	8.7 S	2.4 R	8.2 S	3.5 R	9.0 S	9.0 S	7.0 S	8.2 S	8.3 S	8.3 S	9.0 S	9.0 S	2.3 R
Cf-03	CoJ64	3.8 R	8.8 S	2.3 R	3.4 R	3.4 R	7.2 S	3.2 R	1.2 R	8.7 S	6.4 S	3.5 R	3.8 R	9.0 S	2.5 R
Cf-08	CoJ86	3.3 R	8.1 S	2.3 R	3.4 R	3.5 R	8.3 S	9.0 S	6.2 S	8.6 S	8.5 S	8.8 S	9.0 S	8.8 S	2.3 R

Date of inoculation- 16-08-2021
Date of observation- 16-10-2021
*Score -
**Rating
0 - 4.0 - Resistant (R)
4.1-6 - Intermediate (X)
>6.1 - Susceptible (S).

There is a need to research the emergence of new isolates by adapting new varieties used for commercial cultivation in light of the breakdown of resistance in popular variety that has been observed over the past couple of years.

Efficacy of biocontrol agents against *Colletotrichum falcatum in vitro*

Trichoderma viridae and *Trichoderma harzianum* and *Pseudomonas fluorescens* were tested for their efficacy against *C. falcatum isolates* in vitro (Fig 2). Studies revealed that all the antagonists inhibited or suppressed the mycelial growth of *Colletotrichum falcatum*, out of that *Pseudomonas flourescens* found to be more efficient by showing the inhibition control of 68.18% *in vitro* conditios. (Table 5).

Antagonistic activity of biocontrol agents against *Colletotrichum falcatum in vivo*

The *in vivo* efficiency of all the biocontrol agents in the field condition was studied. As per the healthy control treatment by the fungicide Bavistin (Dip and out sett treatment) was highly effective. The study also indicated that the treatment with Bavistin increased sett germination by 25-60% and reduced rot incidence to certain extent hence selected as control. Pre-treatment of sugarcane setts with antagonist to prevent *C. falcatum* sett entry followed by a set dip in a *C. falcatum* spore suspension reduced ret rot incidence and increased cane yield as compared to the red rot inoculated check (Table 6). Pretreatment of sets with *T. harzianum* and *T. viride* reduce the red rot incidence in comparison to sets without pretreatment. Maximum disease incidence was recorded in the treatment control Studies with no antagonists. Sett treatment with *T. harzianum* caused a significant improvement in cane yield as compared to healthy setts when planted with infected debris.

The treatments with bioagents (healthy setts) had the highest germination rate (60.6%), whereas the treatments with red rot inoculation had the lowest germination rate (20.3%). *Trichoderm*a treatment on pathogen-pre-inoculated setts leads to better germination than the untreated control, with germination rates ranging from 28 to 32.4 per cent.While considering the germination percentage highest reduction in germination was observed in Red rot infected setts but germination failure was substantially lower in cases of *Trichoderma* treatments were used compared to healthy uninoculated setts . Cane mortality ranged from 11.9 to 24.6 per cent in different treatments. Partial chemical control of disease under field conditions might be due to impervious nature of rind and inability of fungicide to reach site of infection in the tissue (Agnihotri,1983, Viswanathan *et al*., 1998). It was concluded that the soil with *T. viride* and *T harzianum* alone or in Control Studies with Bavistin significantly improved the sprouting and plant growth parameters.

Red rot caused by *Colletotrichum falcatum* is the most serious disease of sugarcane affecting cane production in different states in India. However, due to development of new variants of the fungus, new released varieties succumb to the pathogen (Viswanathan 2010, Singh *et al*., 2019) leading to breakdown of resistance necessitating frequent replacement of varieties in different regions of the country. In this context, biological control methods can be used effectively, either in combination or individually as an alternate option. Present study's findings suggest that biocontrol agents *Trichoderma* and *Pseudomonas* show promising antagonistic mechanisms including the induction of resistance, therefore it is reconfirming that bioagents is showing superiority over pathogenic fungi as shown in various crop pathogen systems and our study supports the fact of potential ustulation of bioagents to manage sugarcane red rot disease in Punjab region.

Table 5: Efficacy of antagonists against *C. falcatum* (Dual culture).

Antagonistic organism	Mycelial growth (cm)	Per cent inhibition over control*
Trichoderma viridae	3.3	62.50
Trichoderma harzianum	4.4	50.00
Pseudomonas fluorescens	2.8	68.18

Table 6: Effect of cane treatment with bio-agents on the germination of *Saccharum* spp. complex after 40 days.

Treatment	Variety	% Germination
Healthy control (Bavistin)	CoJ 85	33.20
	CoJ 88	33.40
Disease control (inoculated setts with *Colletotrichum falcatum*)	CoJ 85	21.3
	CoJ 88	20.3
Trichoderma harzianum	CoJ 85	49.96
	CoJ 88	47.96
Trichoderma viridae	CoJ 85	63
	CoJ 88	60.6
Colletotrichum falcatum + *Trichoderma harzianum*	CoJ 85	30
	CoJ 88	28
Colletotrichum falcatum + *Trichoderma viride*	CoJ 85	32.4
	CoJ 88	29.4

CONCLUSION AND FUTURE PERSPECTIVES

Serious threat of red rot of sugarcane against production level will continue throughout the globe. Biocontrol is one of the best option in controlling of this disease and integrated disease management is also good management practice. Biotic agents have the potential to reduce the severity and it will sustain the productivity. Treatment of setts with PGPR/ *Trichoderma* will help to manage the red rot disease of sugarcane. More developed research will be carried out in coming years, there will be depth studies of epidemiology. In addition to this some other strategies which includes to induce systemic resistance against *Collectotrichum,* quarantine regulations, clean cultural practices also should be followed. Transgenic sugarcane development with inbuild red rot resistance will be very good option in future.

Conflict of interest: None.

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