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RESEARCH PAPER

In vitro evaluation of biocontrol agents against *Sclerotium rolfsii* Sacc. causing rootrot of sugarbeet

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Abstract: Eighteen rhizosphere soil samples of healthy sugarbeet plants were collected from sugarbeet growing regions of India for isolation of biocontrol agents. Six *Trichoderma harzianum,* one *T. viride* and sixteen *Bacillus subtilis* isolates were isolated. Efficacy of fungal and bacterial bioagents against *Sclerotium rolfsii* was evaluated by following dual culture technique. Among the *Trichoderma* isolates evaluated, significantly highest inhibition was recorded in *T. harzianum.* Institute of Organic Forming (IOF), University of Agricultural Sciences, Dharwad (UASD) the isolate (69.41 %) followed by *T. viride* Kalparuksha (65.88%). Least inhibition was recorded in *T. viride* isolate TvA5 (57.65 %) followed by *T. harzianum* isolate ThA2 (58.04 %). Among the *B. subtilis* isolates tested, significantly highest inhibition was noticed in *B. subtilis* isolate BsA6 (48.92 %) followed by isolate BsB6 (46. 77 %). Least inhibition was recorded in *B. subtilis* isolate BsA8 (8.06 %) followed by isolate BsA3 (9.14 %).

Key words: Inhibition, Isolate, Root rot, Sugarbeet

Introduction

Sugarbeet (*Beta vulgaris* L.), a member of family *Chenopodiaceae* and native of northern Europe, has become a commercially viable crop for sugar production. Sugarbeets are grown in 60 countries around the world on an area of about 8.7 m ha. Twenty-five per cent of the world's total sugar production is derived from sugarbeet. Sugarbeet root contains 15-20 per cent sucrose and 12-14 per cent recovery is possible in the process of sugar extraction (Kumar *et al*., 2013). A variety of soil borne pathogens affect the roots of adult sugarbeet plants. Among them *Sclerotium rolfsii* Sacc. is the most harmful pathogen causing root rot of sugarbeet resulting in 15-59 per cent reduction in the root yield of various beet cultivars (Mukhopadhyay, 1971).

S. rolfsii is a globally important non-specialized soil-borne fungal pathogen and has a host range of over 500 species. Managing this pathogen by a single method of control through the use of chemicals seems to be a difficult proposition. In recent years, biological control was seen as a possible control strategy against soil-borne plant pathogens. The introduction of antagonists to soil or to the court of infection has achieved significant success (Papavizas and Lewis 1989, Mukhopadhyay and Kaur, 1990). Such antagonists use antibiosis to antagonize pathogens, *i.e.* by generating one or more metabolites that involve antibiotics or other chemicals, mycoparasitis or some type of direct invasion by another organism of a pathogen (Wood and Tveit, 1955). Main objective of the present investigation was isolation of fungal and bacterial biocontrol agents from the rhizosphere soil of sugarbeet and screening them for effectiveness against the root rot pathogen *S. rolfsii* and increasing the productivity of sugar beet cultivation.

Material and methods

Isolation of *Sclerotium rolfsii* Sacc.

The pathogen was isolated from the affected portion of the diseased plants by using tissue segment method (Rangaswami 1958) on sterile potato dextrose medium. The infected plants were pulled out with intact root showing the presence of white mycelial mat with small brown round sclerotia near the collar region were collected and were gently tapped to remove the soil and dirt particle.

Isolation of biocontrol agents from rhizosphere soil of sugarbeet

Collection of soil samples

Eighteen rhizosphere soil samples of healthy sugarbeet plants were collected from sugarbeet crop grown at Amritsar, Punjab (10), Krushi Vigyan Kendra (KVK), Baramati, Maharashtra (6), Indian Institute of Sugarcane Research (IISR), Lucknow, Uttar Pradesh (1) and Main Agricultural Research Station (MARS), UAS, Dharwad, Karnataka (1). Soil samples were collected from different spots in each sampling field and pooled them to get a composite soil sample. The pooled samples were placed in sterile polythene bags, labelled, brought to laboratory and stored in refrigerator at 4 °C before using for isolation of bioagents (Table 1).

Isolation of biocontrol agents

Antagonistic mycoflora and bacteria were isolated by following serial dilution technique (Johnson and Curl, 1977). The soil samples stored in refrigerator were shade dried and then used for serial dilution. To get 10^{-1} dilution, 10 g of soil was dissolved in 100 ml of sterile distilled water, from this 1 ml of soil suspension was taken and added to 9 ml of sterile distilled water to get 10^{-2} dilution. This was repeated until a dilution of 10^{-6} was obtained.

Table 1. Rhizosphere soil samples and isolates of biocontrol agents

Sl. No.	Location	Sample code*	Biocontrol agents isolated			
			Bioagent	Isolate code**	Bioagent	Isolate code***
a) Tarsika (Block), Amritsar (Dist), Punjab						
1	Rasoolpur	AS1	-	-	*B. subtilis*	BsA1
2	Rasoolpur	AS2	*T. harzianum*	ThA2	*B. subtilis*	BsA2
3	Rasoolpur	AS3	-	-	*B. subtilis*	BsA3
4	Rasoolpur	AS4	-	-	-	-
5	Mehnian	AS5	*T. viride*	TvA5	*B. subtilis*	BsA5
6	Rawpur	AS6	-	-	*B. subtilis*	BsA6
7	TalwandiDasondha	AS7	*T. harzianum*	ThA7	*B. subtilis*	BsA7
8	Rawpur	AS8	-	-	*B. subtilis*	BsA8
9	TalwandiDasondha	AS9	-		*B. subtilis*	BsA9
10	Rawpur	AS10	-	-	*B. subtilis*	BsA10
b) Baramati (Taluk), Pune (Dist), Maharashtra						
1	KVK, Baramati	BS1	*T. harzianum*	ThB1	-	-
2	Baramati Rural	BS2	*T. harzianum*	ThB2	*B. subtilis*	BsB2
3	Medad	BS3	-	-	*B. subtilis*	BsB3
4	Malegaon Khurd	BS4	-	-	*B. subtilis*	BsB4
5	Gojubavi	BS5	-	-	*B. subtilis*	BsB5
6	Jalochi	BS6	-	-	*B. subtilis*	BsB6
c) Indian Institute of Sugarcane research, Lucknow, Uttar Pradesh						
1	Lucknow	LS1	*T. harzianum*	ThL1	*B. subtilis*	BsL1
d) Main Agricultural Research Station, UAS, Dharwad, Karnataka						
1	Dharwad	DS1	*T. harzianum*	ThD1	*B. subtilis*	BsD1

Note:
* AS, BS, LS and DS represents the sugarbeet rhizosphere soil samples collected from Amritsar, Baramati, Lucknow and Dharwad, respectively.
** ThA, ThB, ThL and ThD represents the *Trichoderma* isolates isolated from Amritsar, Baramati, Lucknow and Dharwad, soil samples respectively
***BsA, BsB, BsL and BsD represents the *B. subtilis* isolates isolated from Amritsar, Baramati, Lucknow and Dharwad soil samples, respectively.

Antagonistic mycoflora and bacteria were isolated on potato dextrose agar (PDA) and nutrient agar (NA) medium by using a dilution of 10^{-4} and 10^{-6}, respectively. One ml of final dilution of soil suspension was poured into sterilized Petri plates, then the melted and cooled media was poured. Plates were rotated gently on the laminar air flow bench to get uniform distribution of soil suspension in the medium. Then the plates were incubated at 28 ± 2°C and observed at frequent intervals for the development of colonies. Three days old colonies of mycoflora were picked up and purified by hyphal tip method whereas, one day old colonies of bacteria were picked up and purified by streak plate method.

Isolated isolates of *Trichoderma harzianum*, *T. viride* and *Bacillus subtilis* from the soil samples collected from Amritsar, Baramati, Lucknow and Dharwad were designated as ThA, TvA and BsA, ThB, TvB and BsB, ThL, TvL and BsL and ThD, TvD and BsD, respectively.

Identification of fungal and bacterial biocontrol agents

Rhizosphere mycolfora were identified based on mycological keys described by Barnett and Hunter (1972). Whereas, rhizosphere bacteria were identified based on Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Mycoflora were maintained by periodical transfer on to PDA, whereas bacteria were maintained by periodical transfer onto nutrient agar medium.

In vitro* evaluation of biocontrol agents against *S. rolfsii

The bio-efficacy of fungal and bacterial biocontrol agents were evaluated under *in vitro* condition against inhibition of mycelial growth of *S. rolfsii* by dual culture technique (Dennis and Webster, 1971). Along with native antagonists isolated from rhizosphere of sugarbeet, three *Trichoderma* sp. *viz*., *Trichoderma viride* (Trikowin: Microbax (India) Limited) and *T. viride* (Kalparuksha) and *T. harzianum* (Institute of Organic Forming (IOF), UAS, Dharwad) and one *Bacillus subtilis* isolate (IOF, UAS, Dharwad) were also evaluated against *S. rolfsii* under *in vitro* conditions.

Dual culture

Twenty ml of sterilized and cooled potato dextrose agar were poured into sterile Petri plates and allowed to solidify. For evaluation of fungal biocontrol agent, mycelial disc of test fungus *S. rolfsii* was inoculated at one end of the Petri plate and antagonistic fungus were placed opposite to it on the other end. To test the efficacy of antagonistic bacterium a 4 cm line was streaked at one side of plate. On the opposite side to the antagonist mycelial disc of *S. rolfsii* was placed. Three replications were maintained for each treatment with one control maintaining only test fungus. The plates were incubated at 27 ± 1°C and zone of inhibition were recorded by measuring the clear distance between the margin of the test fungus and antagonistic organism. The colony diameter of test fungus in control plate were also recorded.

The per cent inhibition of growth was calculated using the formula given by Vincent (1947).

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

Where,

I = Per cent inhibition of mycelium

C = Growth of mycelium in control

T = Growth of mycelium in treatment

Results and discussion

Six *T. harzianum* isolates *viz*., ThA2, ThA7, ThB1, ThB2, ThL1 and ThD1, one *T. viride* isolate TvA5 and sixteen *Bacillus subtilis* isolates *viz.*, BsA1, BsA2, BsA3, BsA5, BsA6, BsA7, BsA8, BsA9, BsA10, BsB2, BsB3, BsB4, BsB5, BsB6, BsL1 and BsD1 were isolated and identified based on morphological and biochemical characters.

All the native antagonists showed significant reduction in mycelial growth of

S. rolfsii when compared to control. Among the *Trichoderma* isolates evaluated, significantly highest inhibition was recorded in *T. harzianum* IOF isolate (69.41 %) followed by *T. viride* Kalparuksha (65.88 %) and it is on par with *T. harzianum* isolate ThL1(64.71 %) and *T. viride* Trikowin (64.31 %). Least inhibition was recorded in *T. viride* isolate TvA5 (57.65 %) and it is on par with *T. harzianum* isolate ThA2 (58.04 %), *T. harzianum* isolate ThB2 (59.61 %) and *T. harzianum* isolate ThB1 (60.00 %) (Table 2). Among the *B. subtilis* isolates tested, significantly highest inhibition was noticed in *B. subtilis* isolate BsA6 (48.92 %) and it is on par with BsB6 (46. 77 %), BsB4 (44.62 %), BsL1 (44.62 %), BsA10 (44.62 %), BsA9 (44.09 %) and BsB2 (43.55 %) isolates. Least inhibition was recorded in *B. subtilis* isolate BsA8 (8.06 %) and it is on par with isolate BsA3 (9.14 %) (Table 3).

The results are comparable with the findings of Bari *et al*. (2000), Kulkarni (2007), Latha and Rajeswari, (2018) and Ahmad *et al*. (2019). Similar findings were reported by Patel and

Table 2. Inhibition or by *Trichoderma* isolates against *Sclerotium rolfsii* Sacc.

Sl. No.	*Trichoderma* isolates	Per cent inhibition of mycelial growth
1.	*T. harzianum* isolate ThA2	58.04(49.61)*
2.	*T. viride* isolate TvA5	57.65(49.38)
3.	*T. harzianum* isolate ThA7	61.18(51.44)
4.	*T. harzianum* isolate ThB1	60.00(50.75)
5.	*T. harzianum* isolate ThB2	59.61(50.52)
6.	*T. harzianum* isolate ThL1	64.71(53.53)
7	*T. harzianum* isolate ThD1	62.35(52.13)
8.	*T. harzianum* IOF,UASD	69.41(56.40)
9.	*T. viride* Trikowin	64.31(53.30)
10.	*T. viride* Kalparuksha	65.88(54.24)
	S. Em.±	0.45
	C. D. at 1%	1.81

* = Angular transformed values

Table 3. *Effect* of *Bacillus subtilis* (Ehrenberg) Cohn isolation against *Sclerotium rolfsii* Sacc. growth

Sl. No.	*Bacillus subtlis* isolates	Per cent inhibition of mycelial growth
1.	BsA1	18.28(25.30) *
2.	BsA2	43.01(40.97)
3.	BsA3	9.14(17.59)
4.	BsA5	12.90(21.04)
5.	BsA6	48.92(44.37)
6.	BsA7	19.35(26.09)
7.	BsA8	8.06(16.49)
8.	BsA9	44.09(41.59)
9.	BsA10	44.62(41.90)
10.	BsB2	43.55(41.28)
11.	BsB3	34.14(35.74)
12.	BsB4	44.62(41.90)
13.	BsB5	11.29(19.63)
14.	BsB6	46.77(43.13)
15.	BsL1	44.62(41.90)
16.	BsD1	17.74(24.90)
17.	BsIOF, UASD	24.19(29.45)
	S. Em.±	0.73
	C. D. at 1%	2.82

* = Angular transformed values

Anahosur (2001) who observed that *Trichoderma harzianum* coiled the stunted and thickened mycelial strand of *S. rolfsii*, gradually showed sparse growth of *S. rolfsii* and very few sclerotia were formed. Mathur and Sarbhoy (1978) noted *T. viride* and *T. harzianum* as strongly antagonistic bioagents to *S. rolfsii* causing 86.0 per cent inhibition of sugarbeet root rot. Sathri (2000) reported infrequent coiling of *T. harzianum* around *S. rolfsii* hyphae at the point of interaction.

T. harzianum often coiled around the aerial hyphae of *S. rolfsii* and it produces haustoria like structures which enter the mycelium and disorganize the contents of the protoplast and finally lysis of the fungus occurs. This can be attributed because of more competitive ability of *Trichoderma* spp. whether by mycoparasitis, antibiosis or the formation of siderophores (Upadhay and Mukhopadhay, 1983). *Trichoderma spp*. produces secondary metabolites such as antibiotics (6-pentyl-alpha-pyrone (6pp), iso-cyanide derivatives), acids (heptelidic and koningic acid), peptaibols and cell wall degrading enzymes (CDWE) that are involved in the inhibition of radial growth of many plant pathogenic fungi (Fuji *et al.,* 1978 and Vinale *et al.,* 2008).

Conclusion

Results indicated that among the *Trichoderma* isolates evaluated, significantly highest inhibition was recorded in *T. harzianum* IOF-UASD isolate (69.41 %) followed by *T. viride* Kalparuksha (65.88%). Least inhibition was recorded in *T. viride* isolate TvA5 (57.65 %) followed by *T. harzianum* isolate ThA2 (58.04 %). Among the *B. subtilis* isolates tested, significantly highest inhibition was noticed in *B. subtilis* isolate BsA6 (48.92 %) followed by isolate BsB6 (46. 77 %). Least inhibition was recorded in *B. subtilis* isolate BsA8 (8.06 %) followed by isolate BsA3 (9.14 %). Presence of variable degree of effect biocontrol agents in nature provides good scope for development of new bio pesticides.

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