ISSN:0976-4933
Journal of Progressive Science
A Peer-reviewed Research Journal
Vol.15, No.01&02, pp 14-21(2024)

In *vitro* screening of *Trichoderma* species against *Sclerotium rolfsii* Sacc. for antagonism

Subhash Chandra

Department of Botany, Shri Murli Manohar Town P.G. College, Ballia-277001 (U.P.), India

Email: subhashcbsi1@gmail.com

Abstract

In *vitro* study of antagonism was performed by dual culture method between the test pathogen, *Sclerotium rolfsii* Sacc. and different strains of *Trichoderma* species. Maximum inhibition of radial growth of *S. rolfsii* was observed due to *T. harzianum* BHU (90.0%) while no inhibition at all (0.0%) was recorded in case of *T. virens* 2194 and *T. virens* 3067. Based on the results obtained for the colony interaction between different strains of *Trichoderma* species and the test pathogen, five potent strains of *Trichoderma* species were selected for further study. These were as follows: *T. harzianum* BHU, *T. harzianum* IVRI, *T. viride* 1, *T. pseudokoningii* NBRI and *T. virens* BHU. The effect of different concentrations of culture filtrates of the above selected strains of *Trichoderma* species on per cent inhibition of radial growth of *S. rolfsii* was performed. Maximum inhibition of radial growth of *S. rolfsii* was observed in case of *T. harzianum* BHU (44.0%), while minimum inhibition of radial growth was observed by *T. virens* BHU (26.2%) at 40% concentration.

Keywords: In *vitro* screening, *Trichoderma*, *Sclerotium rolfsii* Sacc, Antagonism.

Introduction

The species of genus *Trichoderma* has been reported as most potential biocontrol agents against several soil-borne plant pathogens (Lewis and Papavizas, 1991; Haran *et al*., 1996a; Haran *et al*., 1996b; Elad, 2000; Hermosa *et al*., 2000 Kredics, *et al*., 2003; Joshi, *et al*., 2010; Hermosa, *et al.*, 2012; Keswani *et al*., 2015; Bastakoti *et al.*, 2017; Sridharan *et al*., 2020; Chandra, 2021; 2023) due to their ability to successfully antagonize other fungi. Establishment of the *Trichoderma* and other biocontrol agents in the soil ecosystem has greatly affected by numerous biotic (nature of the target organism and of the host plant, presence of predators, parasites or antagonistic microorganisms among the resident micro flora) and abiotic (nature of the soil or substrate, humidity, availability of nutrients, temperature, radiations, salinity and pH) factors (Dandurand and Knudsen, 1993; Eastburn and Butler, 1988a, b; Hubbard *et al*., 1983; Knudsen and Bin, 1990). There are several mechanisms involved in antagonism of *Trichoderma* species namely antibiosis, enzyme secretion, substrate competition, hyphal interactions and mycoparasitism (Haran *et al*., 1996b). In order to solve the national and global problems of environmental hazards due to application of chemicals for disease control, antagonistic microbes have been considered as prospective agents for the purpose (Cook, 1985). Chemicals are necessary for control of different diseases but its

adverse effect on human and animal health, environmental contamination, phytotoxicity, development of resistance against pathogens, and their high cost (Mulder, 1979; Mukherjee and Garg, 1983) make their application difficult to be continued in future.

Pesticides were originally based on toxic heavy metals such as arsenic, mercury, lead or copper. Modern pesticides being organic compounds, with a high degree of specificity towards their target organism, also exert effects on non-target beneficial organisms and consequently may be hazardous threat to the environment. It is also possible that long-term effects of these compounds might be subtly detrimental to soil fertility. Moreover, the practical usefulness of these measures in the tropics is rather doubtful, as it is being coherent to the development of resistant mutants of the pathogen concomitant with the prevalence of new pathological races. These fears have led biotechnologists to examine alternatives to chemical pesticides as a means of controlling agricultural pests. A relative alternative for such environmental pollution is to include integration of biological control for control of mites, nematodes, plant pathogens and weeds (Tauber and Baker, 1988). Therefore, biological control of plant diseases has received significant attention, since it promises to offer a more sustainable food supply. Moreover, a successful biological management strategy of a crop disease can offer a marketable products at considerably lower cost compared to conventional measures (Chung, 1994).

The pathogen *Sclerotium rolfsii* Sacc. is an important stem (sudden wilting or flagging on one or more shoots) disease pathogen and responsible for southern stem blight of Soybean. The pathogen is responsible for causing destructive diseases of many economically important crops including vegetables and field crops (Singh, 1974; Khan and Kolte 1989). A wide host range of the pathogen has been recorded covering more than 500 species of cultivated and wild plants in tropical, subtropical and warm regions of the world (Aycock, 1966; Punja, 1985). The pathogen survives as clusters of brown, round sclerotia, which are about the size of mustard seeds, each with a diameter of 0.8-2.5 mm are often scattered on the surface of the soil or on the lower stem or collar of the dying plant. The sclerotia serve as the source of primary inoculum and usually, produced the superficial, white, fan-like fungal growth mat on the soil surface around the base or lower stem of the wilted plants as well as on fallen leaves and other organic debris. In the present study, twelve strains of six *Trichoderma* species were used as antagonists to screen out the effective antagonistic strains against *Sclerotium rolfsii* Sacc., the causal agent of southern stem blight disease of Soybean.

Materials and methods

Source of the pathogen

A virulent strain of *Sclerotium rolfsii* Sacc. was obtained from the Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. The culture was maintained on Potato-Dextrose Agar medium at 25± 2 ^{o}C by regular subculturings.

Source of the *Trichoderma* species

The pure culture of different strains of *Trichoderma* species were obtained from Laboratory of Applied Mycology and Plant Pathology, Department of Botany, Banaras Hindu University, Varanasi where the cultures were maintained from the collection centres of Institute of Microbial Technology (Chandigarh), National Botanical Research Institute (Lucknow), Indian Agricultural Research Institute (New Delhi), Indian Vegetable Research Institute (Varanasi). Local species/strains of *Trichoderma* were isolated from soils of various locations from and around Banaras Hindu University Campus, Varanasi, on the

Trichoderma Selective Medium. The cultures were maintained on PDA by periodically subculturing and were stored in a refrigerator at 4 ^{o}C.

Performance of Pathogenicity Test

Seed of the susceptible variety of Soybean (NRC 7) was surface sterilized with 0.1% NaOCl solution for 1 min, washed thoroughly with sterilized distilled water and sown in earthenware pots (15 × 25 cm size) containing sterilized garden soil. The Pathogenicity test of *Sclerotium rolfsii* Sacc. was performed in pure sand inoculum of the test pathogen mixed with sterilized garden soil in the ratio of 1:9. The sand inoculum of the test pathogen was prepared in sand + 3% maize meal (Upadhyay and Rai, 1987). The seedlings (12-day old) were transferred to inoculum of soil mixture. The moisture level of the soil was maintained at 15-20%. The pots were kept in polyhouse and process of disease development was observed. Infected stems were collected, washed and cut into small pieces. They were treated with 0.1% NaOCl for 1 min, rewashed with sterilized distilled water and transferred on Potato Dextrose Agar (PDA) in slants. After 7 days, the isolated organism was examined, compared with the original stock cultures and their identity was confirmed.

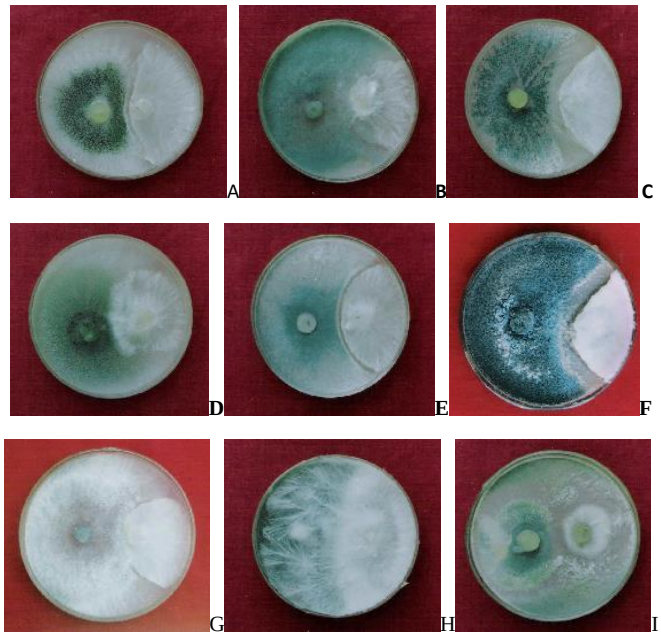

Figure 1. *In vitro* screening of different *Trichoderma* species against *S. rolfsii*

A. ***T. harzianum* NBRI**	B. ***T. virens* BHU**	C. ***T. harzianum* BHU**
D. ***T. pseudokoningii* NBRI**	E. ***T. harzianum* IVRI**	F. ***T. koningii* NBRI**
G. ***T. viride* 1**	H. ***T. virens* 3067**	I. ***T. atroviride* BHU**

***In vitro* screening of *Trichoderma* species against *Sclerotium rolfsii* Sacc. for antagonism**

Colony interactions

The colony interaction was studied in dual culture following the method described by Upadhyay and Rai (1987). Five mm agar blocks of freshly grown culture of *Sclerotium rolfsii* Sacc. from the margin of the colony were placed separately with different strains of *Trichoderma* species over PDA medium 3 cm apart in paired combinations. The inoculated plates were incubated at 25 ± 2^{o}C for 6 days. The control

sets were single or dually inoculated cultures of the same fungus. The colony interactions were assayed as per cent inhibition of the radial growth of the pathogen following the formula: $R_1 - R_2 / R_1 \times 100$ (Fokkema, 1976), where, R_1 denotes the radial growth of the pathogen towards the opposite side and R_2 denotes the radial growth of the pathogen towards the test antagonist.

Inhibitory effect of the culture filtrates of selected *Trichoderma* species on radial growth of *Sclerotium rolfsii* Sacc.

Based on the results obtained in colony interaction between different strains of *Trichoderma* species and the test pathogen, five potent strains of *Trichoderma* species were selected for further study. These were as follows: *T. harzianum* BHU, *T. harzianum* IVRI, *T. viride* 1, *T. pseudokoningii* NBRI and *T. virens* BHU.

The selected antagonists and the test pathogen were grown on PDA medium in Petri dishes at 25 ± 2 ^{o}C for 4 days. Two blocks of the equal size (5 mm each) cut from the margins of actively growing cultures of *Trichoderma* species, were inoculated separately into 250 ml Erlenmeyer flask each containing 100 ml sterilized potato dextrose broth in triplicate. The flasks were then incubated at 25 ± 2 ^{o}C for 10 days after which the culture filtrates were filtered through Whatman filter paper no. 44 and finally through a Seitz filter (G 4) attached to vacuum pump to obtain filter sterilized cell free culture filtrates. Two, four and eight ml culture filtrates of each *Trichoderma* species were poured into empty sterilized plates in three replicates which was immediately followed by pouring 18, 16 and 12 ml of autoclaved and cooled PDA medium, so as to make the final concentration of culture filtrates 10, 20 and 40%, respectively. Five mm agar blocks of actively growing 5-day old culture of the test pathogen were cut from the margin and inoculated at the center of Petri-Plate separately containing potato dextrose agar (PDA) medium and the culture filtrate of *Trichoderma* species. The control set was made by pouring 20 ml PDA medium only in sterilized Petri plates. The inoculated Petri plates were incubated at 25°C and measurement of the radial growth was done after 4 days of incubation. The percent inhibition in the radial growth was calculated by using the formula: Per cent growth inhibition = C – T /C ×100, where, C = Radial growth in control set; T = Radial growth in treated set.

Results

***In vitro* screening of *Trichoderma* species against *Sclerotium rolfsii* Sacc.**

Colony interactions

The results of the colony interaction between the test pathogen and different strains of *Trichoderma* species is presented in Table 1. Twelve strains of six *Trichoderma* species were screened for their antagonistic activity against *S. rolfsii in vitro* by dual culture technique (Figures 1).

Maximum inhibition of radial growth of *S. rolfsii* was observed due to *T. harzianum* BHU (90.0%) while no inhibition at all (0.0%) was recorded in case of *T. virens* 2194 and *T. virens* 3067 (Table 1). Two strains of *T. viride* (*T. viride* 1433 and *T. viride* 2109) showed less than 30% inhibition of radial growth of *S. rolfsii*.

Table1. In vitro screening of *Trichoderma* species against test pathogen (*Sclerotium rolfsii*)

Antagonists	Test Pathogen (% inhibition) * (*Sclerotium rolfsii*)

T. harzianum BHU	**90.0± 0.83**
T. harzianum NBRI	53.9± 0.96
T. harzianum IVRI	75.0± 0.79
T. viride 2109	25.6± 0.61
T. viride 1433	18.4± 0.76
T. viride 1	61.9± 0.45
T. koningi NBRI	70.7± 0.72
T. pseudokoningi NBRI	76.8± 0.52
T. virens BHU	76.8± 0.52
T. virens 3067	00.0± 0.00
T. virens 2194	00.0± 0.00
T. atroviride BHU	52.1± 0.72

*Values are average of three replicates ± SEM

Table 2. Effect of culture filtrate of selected strains of *Trichoderma* species on the per cent inhibition of radial growth of *Sclerotium rolfsii*

***Trichoderma* species**	***Sclerotium rolfsii* (Concentration in per cent*)**		
	10	**20**	**40**
T. harzianum BHU	34.2±0.4	38.6±0.6	**44.0±0.2**
T. harzianum IVRI	28.1±0.6	30.2±0.5	33.1±0.4
T. viride 1	21.2±0.1	26.4±0.3	34.5±0.5
T. pseudokoningi NBRI	32.6±0.3	34.2±0.4	37.1±0.5
T. virens BHU	19.8±0.4	21.7±0.5	26.2±0.3

*Values are average of three replicates ± SEM

Based on the results obtained for the colony interaction between different strains of *Trichoderma* species and the test pathogen, five potent strains of *Trichoderma* species were selected for further study. These were as follows: *T. harzianum* BHU, *T. harzianum* IVRI, *T. viride* 1, *T. pseudokoningii* NBRI and *T. virens* BHU.

Effect of culture filtrates

The effect of different concentrations of culture filtrates of the above selected strains of *Trichoderma* species on per cent inhibition of radial growth of *S. rolfsii* was presented in Table 2. Three concentrations of culture filtrates such as 10%, 20% and 40% were used in the present study. The level of inhibition in radial growth of the test pathogen was increased with increasing the concentrations of the culture filtrate. Maximum inhibition in radial growth of *S. rolfsii* was observed at 40% concentration in case of *T. harzianum* BHU (44.0%) which was followed by *T. pseudokoningii* NBRI (37.1%) and *T. virens* BHU (34.5%).

Discussion

In *in vitro* experiment, all the strains of *Trichoderma* species, except *T. virens* 2194 and *T. virens* 3067, showed varied degree of inhibition on the radial growth of the *S. rolfsii*. The maximum inhibition of the growth of *S. rolfsii* was observed due to *T. harzianum* BHU (Table 1). The *in vitro* antagonistic effect of *Trichoderma* species was reported long back (Dennis and Webster, 1971 a, b; Wells *et al*., 1972; Chet *et al.*, 1978). *Trichoderma* spp. directly attack and lyse the mycelium and sclerotia of *Sclerotium rolfsii* (Upadhyay and Mukhopadhyay, 1986; Singh and Dwivedi, 1987).

The interaction of the antagonists and the pathogen and occurrence of inhibition zone on agar media could be commonly considered as a result of the production of antibiotics and competition for nutrients and space as observed by Upadhyay and Rai (1987). The growth inhibition of a fungus *in vitro* by another cannot be expected to be same in field soil because of different ecological factors. However, *in vitro* studies might indicate the potentiality of antagonism of a fungus in soil. Such studies are important for screening the effective antagonists against soil-borne pathogens (Bells *et al*., 1982).

The effect of culture filtrates of the potent strains of *Trichoderma* species showed varied degree of inhibitory effect on *S. rolfsii* depending on their concentration and toxicity produced by them. In present study maximum inhibition in radial growth of *Sclerotium rolfsii* was recorded with *T. harzianum* BHU at all the tested concentration while minimum reduction was recorded with *T. virens* BHU (Table 2).

Acknowledgement

Author is thankful to Late Professor Bharat Rai, Department of Botany, Banaras Hindu University Varanasi, India for providing necessary facilities and valuable supervision during the course of study.

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Received on 23.3.2024 and accepted on 18.05.2024