

## ***In vitro* antifungal potential of rhizospheric isolates against *Fusarium oxysporum* causing Fusarium wilt of Bt-cotton**

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### **ABSTRACT**

Fusarium wilt is one of the drastic diseases of Bt-cotton caused by *Fusarium oxysporum f.sp. vasinfectum*. In order to find effective measure to control this phytopathogen, 114 rhizospheric isolates were screened for *in vitro* antifungal potential against *Fusarium oxysporum f.sp. vasinfectum* using dual culture method. Out of 114 isolates tested, 13 rhizospheric isolates inhibited growth of *Fusarium oxysporum f.sp. vasinfectum in vitro*. Highest antifungal activity was shown by isolate RLS19 (68.89 %) and followed by RLS52 (62.22 %), RLS53 (60.00%), RLS72 (57.78 %), and RLS101 (57.78 %) in dual culture technique against phytopathogen. Characterization of microbial control mechanism, rhizospheric isolates was evaluated by testing the production of volatile metabolites, diffusible metabolites and siderophore production. Rhizospheric isolates was isolate RLS101 inhibit 50 % of growth followed by RLS 52 (20 %) and RLS79 (10 %) by producing volatile metabolites against phytopathogen others were unable produce volatile metabolites. Diffusible metabolites was produced by rhizospheric isolates RLS19, RLS52 and RLS18 which inhibits maximum growth phytopathogen 57.50 %, 55.00 % and 52.50 % respectively. Qualitative siderophore production was detected by modified CAS agar assay method, 8 rhizospheric isolates were able to produce siderophore and highest activity was shown by RLS18, RLS53 and RLS58.

**KEY WORDS:** BT-COTTON, *FUSARIUM OXYSPORUM F.SP. VASINFECTUM*, VOLATILE METABOLITES, DIFFUSIBLE METABOLITES, SIDEROPHORE

### **ARTICLE INFORMATION:**

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Received 10<sup>th</sup> June, 2016

Accepted after revision 25<sup>th</sup> June, 2016

BBRC Print ISSN: 0974-6455

Online ISSN: 2321-4007



Thomson Reuters ISI SCI Indexed Journal

NAAS Journal Score : 3.48

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Online Contents Available at: <http://www.bbrc.in/>

## INTRODUCTION

Bt- cotton is one of the most important cash crops cultivated in India. In north zone of India, Maharashtra is state first rank in cultivation area but trails on third rank for production (current cotton scenario, CCI-2013-14) due to different environmental factors and attack of pests. Fusarium wilt is one of major destructive and yield reducing diseases of Bt-cotton caused by the fungal phytopathogen *Fusarium oxysporum* f.sp. *vasinfectum* (Atkinson, 1892). All growth stages of Bt-cotton are susceptible to Fusarium wilt disease and widespread in most cotton-growing countries of the world. This disease was first reported in India (Kulkarni, 1934) and spread through infected soil and seed (Bennett *et al.*, 2008).

Present control strategies to control the Fusarium wilt disease is by using chemical pesticides. Chemical control measures create imbalances in the microbial community, which may be unfavorable to the activity of beneficial organisms and could lead to the development of resistant strains of pathogen and pollution of environment. Since, Fusarium wilt is increasingly destructive in cotton production, and to avoid the hazardous effect of chemicals new ways of alternative controlling the diseases need to be searched. The production of siderophores in rhizosphere fungi was higher than those isolated from the contaminated soil. Siderophore production by Fungal M10 strain was studied using CAS blue agar and the iron(III)-chelating compounds, excreted by the microorganism and diffused through the medium producing a colour change from blue to orange. Microbial siderophores production increases virulence power of microorganisms and serve as good biocontrol agents, (Talebi *et al.*, 2011, Hussein and Joo, 2012, Vinale *et al.*, 2013 and Balado *et al.* 2015).

Microbiological control of plant pathogen by rhizospheric microorganisms offers an attractive alternative over chemical control. There are evidences that microorganism can be served as good microbial control candidate to suppress the diseases. U.S. Environmental Protection Agency registered eight species of microorganisms for commercial use against soil borne plant pathogens in the United States (Cook *et al.*, 1996). Researchers from different region reported microorganisms as biocontrol agents such as *Pseudomonas sp.* (Chernin *et al.*, 2011 Sandheep *et al.*, 2012; ), *Bacillus sp.* (Saha *et al.*, 2012; Lamsal *et al.*, 2012), *Trichoderma sp.* ( Ahith and Lakshmi-devi, 2010; Otadoh *et al.*, 2011; Sandheep *et al.*, 2012) *Serratia sp* (Chernin *et al.*, 2011) etc. Several researchers working on biological control of *Fusarium* wilt of different crops but there is less literature available on microbiological control of Fusarium wilt of Bt- cotton. Biological Control of *Fusarium* Wilt of Cotton was studied by using endophytic bacteria (Chen *et al.* 1995),

*Trichoderma sp* (Sivan and Chet, 2008 and Mali and Ramaiah., 2015).

Soil serves as excellent culture medium for all types of microorganisms due to the abundant availability of nutrient and favorable environmental condition. Rhizosphere of plants has been frequently exploited as brilliant source for searching microbial control agents. It has been suggested that microorganisms isolated from the rhizosphere of a particular crop may be better adapted to that crops rhizospheric environment and may provide better control of diseases than the other plant rhizosphere species (Cook, 1993). The present study was aimed to i) Isolation of phytopathogen responsible for causing *Fusarium* wilt of Bt-cotton, ii) isolation of rhizospheric isolates from healthy Bt-cotton plant iii) *in vitro* screening of rhizospheric isolates for antifungal activity against *Fusarium* wilt pathogen and (iii) characterization of *in vitro* microbial control mechanism of efficient rhizospheric isolates by testing for production of volatile metabolites, diffusible metabolites and siderophore.

## MATERIAL AND METHODS

### ISOLATION OF PHYTOPATHOGEN

*Fusarium* wilt infected Bt- cotton plants were collected from different fields of Beed, Hingoli and Vasanttrao Naik Agricultural University, Parbhani district in sterile polyethylene bags and brought to research laboratory, Department microbiology Yogeshwari Mahavidyalaya, Ambajogai. Infected stems and branches of Bt-cotton were washed with sterile water thoroughly. The infected stem tissues were cut into small pieces (2–5 mm size) and by using flame-sterilized forceps; they were transferred to sterile Petri plates containing 0.1 % mercuric chloride solution for 30-60 s. These surface sterilized pieces of stem tissues are again transferred to another sterile Petri plates containing sterile distilled water to remove the disinfectant solution with 2-3 changes of sterile water. The outer layer of tissues were removed rapidly with sterile surgical blade and small pieces from the central core of tissues in the advancing margin of infection are cut and aseptically transferred to Petri plates containing Potato Dextrose Agar (PDA). Petri plates are incubated at room temperature for 6 days (Narayanasamy, 2011). Isolated pure culture of phytopathogen was maintained on PDA and Kings- B agar plate and also on slant for further use.

### ISOLATION OF RHIZOSPHERIC BACTERIA

Rhizospheric soil samples from healthy Bt- cotton growing fields of different districts of Marathwada region (Beed, Latur, Osmanabad, Aurangabad, Jalna, Nanded, Hingoli and Parbhani) were collected in sterile polyethyl-

ene bags and brought to research laboratory. 1g of each rhizospheric soil sample was mixed with 100 ml sterile distilled water and shaken well for 2 min, and then the content of flask was allowed to settle. Different dilutions  $10^{-1}$ , to  $10^{-6}$  of these samples were prepared by serial dilution technique. Highest dilution  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  dilutions used for plating propose. 0.5 ml selected dilutions was plated by using pour plate technique. All the plates were incubated at room temperature for 24-48 h.

### IN VITRO SCREENING FOR MICROBIAL CONTROL AGENT

114 rhizobacteria isolates were screened for antifungal activity against *Fusarium oxysporum* f.sp. *vasinfectum* by using dual culture technique on King B agar plates (Gull and Hafeez, 2012). 5 mm diameter mycelial disc was punched from margin of actively growing mycelium and placed at the center of 9 cm Petri plate and rhizobacterial isolates were inoculated 3 cm apart from the center. Three rhizospheric isolates were placed in a plate along with phytopathogen at the center. Control plate was kept without inoculation of rhizobacteria isolates and the all the plates were incubated at 28 °C for 6 days. The antifungal activity was determined by measuring the inhibition of mycelial growth of *Fusarium oxysporum* f. sp. *vasinfectum*.

### MECHANISM OF MICROBIAL CONTROL AGENT

To characterize the mechanism of microbial control agent, the efficient rhizospheric isolates were tested for the production of volatile metabolites, diffusible metabolites and siderophore.

### DETECTION OF VOLATILE METABOLITES

Volatile metabolites detection of efficient rhizospheric isolates was done by using double plate method (Dennis and Webster, 1971). The kings-B agar plate inoculated with a 5 mm mycelial disc of *Fusarium oxysporum* f.sp. *vasinfectum* and rhizospheric isolate at the center of 90 mm Petri plate separately. Both the inoculated plates were placed facing each other and sealed with cellophane adhesive tape. Control was kept without inoculation of rhizospheric isolate. Procedure repeated for each efficient rhizospheric isolates in triplicates. All the plates were incubated at 28 °C for 6 days. The production of volatile metabolites was then determined by inhibition of *Fusarium oxysporum* f.sp. *vasinfectum* and percentage of radial growth inhibition was calculated by using the formula (Whipps, 1987).

$$PI = \frac{R1-R2}{R1} \times 100$$

Where, R1 is radial growth of the pathogen alone (a control value) and R2 is radial growth pathogen in the direction of the antagonist pathogen + antagonist (an inhibition value).

### DETECTION OF DIFFUSIBLE METABOLITES

Diffusible antifungal metabolites were detected by well diffusion assay (Schlumbaum *et al.*, 1986). Rhizobacterial isolates showing antifungal activity against *Fusarium oxysporum* f.sp. *vasinfectum* during screening were grown in King B broth at room temperature on rotary shaker at 150 rpm for 48 h to obtain cell free culture filtrate. Kings B agar plates were prepared and after solidification with the help of sterile cork borer three wells 3cm apart from the center 90 mm diameter were punched on a plate. These wells were labeled according to the rhizobacteria isolates cell free culture filtrate to be loaded. 5 mm plugs from leading edge of 3 day old culture *Fusarium oxysporum* f.sp. *vasinfectum* were punched and kept at the center of the plate. Different rhizobacterial isolate broths were filtered by using Millipore syringe filter 0.22  $\mu$  (Hi-media) to prepare filtrate for diffusible metabolites. Each well was loaded with 100  $\mu$ l cell free culture filtrate aseptically. Control was kept without inoculation of rhizobacterial cell free culture filtrate. All the plates were incubated at 28 °C for 6 days. Plates were observed for zone of inhibition. Percentage of radial growth inhibition of pathogen was calculated by using the formula, (Whipps, 1987).

### QUALITATIVE DETECTION OF SIDEROPHORE

Siderophore production was determined by using modified Chrome Azurol S (CAS) assay (Milagres *et al.*, 1999). Initially all the glassware's are rinsed with distilled water and dried. 60.5 mg of CAS was weighed accurately and dissolved in 50 ml of distilled water and to this add 10 ml of iron solution (1 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , in 10 mM HCl). 72.9 mg of Hexa decyl tri methyl-ammonium bromide (HDTMA) dissolved in 40 ml distilled water. CAS and Iron solution mixture was slowly added to 40 ml HDTMA with constant stirring to obtain dark blue colour. Basal Medium containing 30.24 g Pipes, and 12 g of a 50 % (w/w) NaOH to rise the pH to the pKa of Pipes (6.8) and 15 g Agar in 750 ml distilled water. All the contents are separately sterilized by autoclaving at 121 °C for 15 min. After cooling to 50 °C to basal medium 750 ml add 100 ml CAS-Fe-HDTMA mixture along the glass wall and agitated with enough care to avoid foaming. Petri dishes (9.0 cm in diameter) were prepared with 30 ml of Nutrient agar medium for culturing rhizospheric isolates. After solidification, the medium was cut into halves, one of which was replaced by CAS blue agar (15 ml) and allowed to solidify. The halves containing

culture medium was inoculated with 24 h old rhizospheric isolate near the borderline of the two medium. The same procedure was repeated for each rhizospheric isolate. Un-inoculated CAS agar plate serves as control. All plates were incubated at room temperature for 5 days and changed CAS agar colour from blue to orange or purple or dark purplish- red indicates the siderophore production.

## RESULTS AND DISCUSSION

### ISOLATION OF PHYTOPATHOGEN

*Fusarium* wilt phytopathogen was isolated from infected Bt-cotton plant. The phytopathogen produced whitish mycelial growth on inoculated PDA plates and growth of fungal mycelium was transferred on fresh PDA and purified (Fig.1). Based on macro and microscopic observations of mycelial growth showed boat-shaped macroconidia, with slightly tapering apical cells and hooked basal cells 4-celled, microconidia ellipsoidal, 1-celled and chlamydospores are globose, usually solitary, phytopathogen was identified as species *Fusarium oxysporum* (Watanabe, 2010;

Gull and Hafeez, 2012). The phytopathogen was transferred on fresh PDA and Kings B agar plates and also on maintained on slants for further use.

### ISOLATION OF RHIZOSPHERIC BACTERIA

114 rhizobacterial isolates from different Bt-cotton fields were isolated from rhizospheric soil samples by serial dilution technique using pour plate method . Isolates were selected on the basis of distinctive morphology, size, shape and colour of the bacterial colony and location of the sample. All the isolates were tentatively labeled as RLS01 to RLS114 and maintained on nutrient agar slants with periodic transfer to fresh medium for future applications.

### IN VITRO SCREENING FOR MICROBIAL CONTROL AGENT

All the 114 rhizospheric isolates were screened for in vitro antifungal activity against *Fusarium oxysporum* f.sp. *vasinfectum* by dual culture technique Gull and Hafeez, 2012). Out of 114, 13 rhizobacterial isolates inhibited mycelial growth in dual culture. These isolates showed significant differences in mycelial growth inhibition. Radial growth of phytopathogen in test and control was measured and recorded (Table 1 and Fig 2). Rhizospheric isolates showed significant inhibition RLS19 (68.89 %), RLS52 (62.22 %), RLS53 (60.00 %), RLS72 (57.78 %) and RLS101 (57.78 %) of *Fusarium oxysporum* f.sp. *vasin-*

*fectum* in dual culture technique. The highest antifungal activity was shown by rhizospheric isolate RLS19 (68.89 %) and followed by RLS52 (62.22 %), RLS53 (60.00 %).

Similar results were also recorded by other investigators, rhizospheric *Pseudomonas* isolate, isolated from rhizospheric soil of tomato evaluated against *Fusarium oxysporum* f.sp. *lycopersici* by dual culture technique and maximum zone inhibition was 22 mm (Asha *et al.*, 2011). Rhizospheric isolates, *Pseudomonas sp.* and *Bacillus sp.* were evaluated for biocontrol potential against *Fusarium oxysporum* f.sp. *ciceris* by dual culture technique, where *Bacillus subtilis* B28 showed 51.16 % inhibition (Karimi *et al.*, 2012). Endophytic bacteria were antagonistic against *F. oxysporum*, isolates EB1 and EB2 inhibits radial growth 42.60 % and 41.00 % respectively in dual culture test (Edward *et al.*, 2013). Soil isolates SC09-21, SG09-01, SR04-02 and SR04-16 inhibited the mycelial growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici* *in vitro* by dual culture technique. Isolate SC09-21 showed 12.2 mm and SG09-01, R04-02 and SR04-16 less than 10 mm zone of inhibition due to antibiosis (Xu and Kim, 2014). *Trichoderma harzianum* significantly reduce the mycelial growth of *Fusarium oxysporum* f.sp. *ciceris*, a wilt pathogen of chickpea ranging from 20.11% to 65.78% (Srivastava *et al.*, 2015).

When results of previous researchers (Xu and Kim, 2014; Asha *et al.*, 2011; Karimi *et al.*, 2012; Edward *et al.*, 2013; Xu and Kim, 2014; Srivastava *et al.*, 2015) compared to our findings, our finding found better than the others, where rhizospheric isolates RLS19 (68.89 %), RLS52 (62.22 %), RLS53 (60.00%) RLS72 (57.78 %), and RLS101 (57.78 %) found better in inhibiting the *Fusarium sp.* Based on screening results in dual culture technique, eight isolates namely (RLS18, RLS19, RLS52, RLS53, RLS58, RLS72, RLS76 and RLS102) with good inhibition activities of *Fusarium oxysporum* f.sp. *vasinfectum* were selected for further study.

### MECHANISM OF MICROBIAL CONTROL AGENT

While finding the mechanism of the microbial control agent, the rhizospheric isolates were tested for the production of volatile metabolite, diffusible metabolite and siderophore production.

### DETECTION OF VOLATILE METABOLITES

After 6 days incubation it was observed that three rhizospheric isolates were able to produce volatile metabolites and inhibit the radial growth of *Fusarium oxysporum* f. sp. *vasinfectum* (Fig. 3 and Fig. 4). Highest Inhibition of phytopathogen by producing volatile metabolites was shown by rhizospheric isolate RLS101 (50 %) followed by RLS52 (20 %) and RLS79 (10 %).



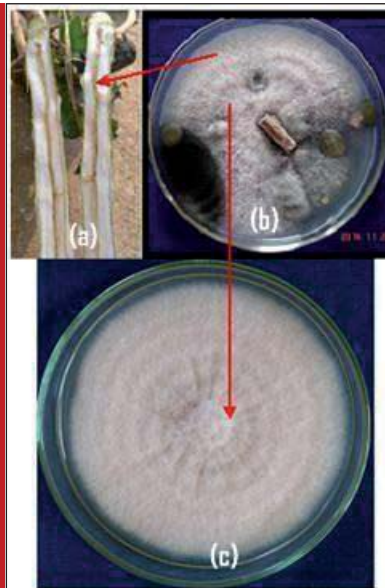


FIGURE 1: Isolation of *Fusarium* wilt causing phytopathogen from infected Bt-cotton stem (a) infected cotton stem (b) isolation of wilt pathogen from infected cotton stem (c) isolated Phytopathogen

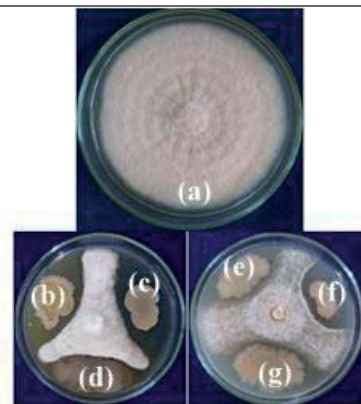


FIGURE 2: In vitro screening of antifungal activity of rhizospheric isolates against *Fusarium oxysporum* f.sp. *vasinfectum* (a) Control (b) RLS19 (c) RLS107 (d) RLS58 (e) RLS72 (f) RLS53 and (g) RLS52

Other isolates were unable produce volatile metabolites and there was no inhibition of *Fusarium oxysporum* f.sp. *vasinfectum* growth. Previous researcher found that *Pseudomonas aeruginosa* P12 isolate inhibits 26.30 % radial growth of *Fusarium oxysporum* f. sp. *ciceris* by producing volatile metabolites (Karimi *et al.*, 2012). This

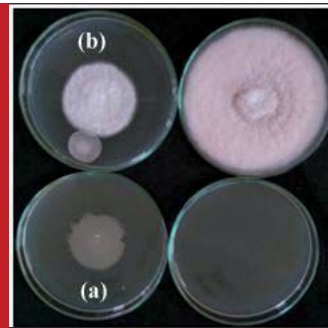


FIGURE 3: Detection of volatile metabolite production of rhizospheric isolate RLS102 against *Fusarium oxysporum* f.sp. *vasinfectum* (a) Control (b) Test

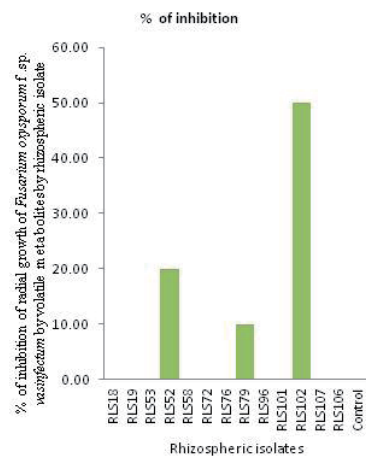


FIGURE 4: Production of volatile antifungal metabolites of rhizospheric isolates against *Fusarium oxysporum* f.sp. *vasinfectum*

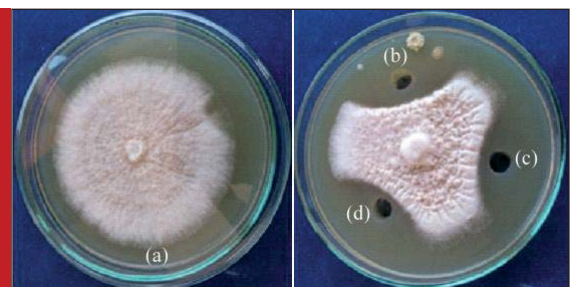


FIGURE 5: Detection of diffusible metabolites production of rhizospheric isolate by agar well diffusion method against *Fusarium oxysporum* f.sp. *vasinfectum* (a) Control (b) RLS18 (c) RLS76 (d) RL19

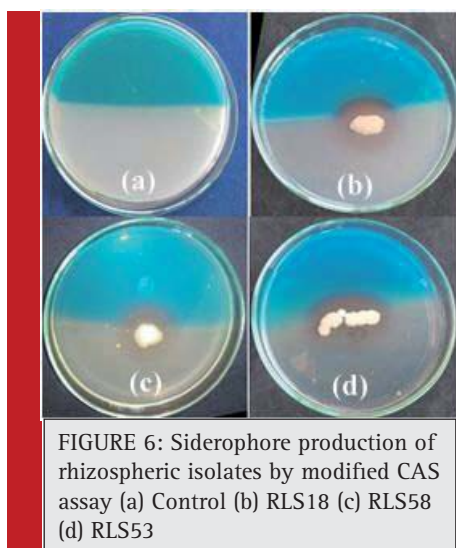


FIGURE 6: Siderophore production of rhizospheric isolates by modified CAS assay (a) Control (b) RLS18 (c) RLS58 (d) RLS53

finding suggests that volatile metabolite production by rhizospheric isolates is one of the mechanism by which phytopathogen can be controlled. Here in present study, rhizospheric isolate RLS101 found better in controlling *Fusarium oxysporum* f.sp. *vasinfectum* by producing volatile metabolites than *Pseudomonas aueruginosa* P12 (Karimi *et al.*, 2012).

### DETECTION OF DIFFUSIBLE METABOLITES

Diffusible antifungal metabolites were studied by well diffusion assay (Schlumbaum *et al.*, 1986). Eight efficient rhizospheric isolates tested showed inhibitory

effect on *Fusarium oxysporum* f.sp. *vasinfectum* by producing diffusible metabolites (Fig. 5 and Table 2). Rhizospheric isolate RLS19 (57.50 %) showed highest inhibition of phytopathogen by producing diffusible metabolites followed by RLS52 (55.00 %) and RLS 18 (52.05 %). *Bacillus subtilis* B28 isolate also inhibiting *Fusarium oxysporum* f.sp. *ciceris* (78.30 %) by producing diffusible metabolites (Karimi *et al.*, 2012).

### QUALITATIVE DETECTION OF SIDEROPHORE

After 5 day incubation, eight isolates produced siderophore on modified CAS plate. Siderophore production was recorded in the form of grades (Fig.6 and Table 3). The highest siderophore production was recorded in the form of change in colour of the medium from blue to purple or orange (Fig. 6). Highest siderophore producing rhizospheric isolates were RLS18, RLS53 and RLS58. Siderophore production was recorded in the form of change in colour from blue to purple or orange (Milagres *et al.*, 1999). CAS agar plate assay indicated that all the rhizobacterial isolates have siderophore production ability, RLS18, RLS53 and RLS58 found superior compared to other rhizospheric isolates. Similar result was reported by other researchers (Chaiharan *et al.*, 2009). The qualitative production of siderophore by *B. cereus* with orange halos during the exponential growth and Sporulation phases by modified CAS plate assay (Laloo *et al.*, 2010).

Production of siderophores by different fungal species isolated from heavy metal contaminated and

Table 1: *In vitro* screening of antifungal activity of rhizospheric isolates against *Fusarium oxysporum* f.sp. *vasinfectum*

Sr. No.	Rhizospheric isolate	Growth towards antagonist in mm (R2)	Growth of control in mm (R1)	% of inhibition
1	RLS18	20	45	55.56
2	RLS19	14	45	68.89
3	RLS53	18	45	60.00
4	RLS52	17	45	62.22
5	RLS58	20	45	55.56
6	RLS72	19	45	57.78
7	RLS76	20	45	55.56
8	RLS79	22	45	51.11
9	RLS96	25	45	44.44
10	RLS101	23	45	48.89
11	RLS102	22	45	51.11
12	RLS107	19	45	57.78
13	RLS106	22	45	51.11
14	Control		45	100.00

Table 2: Production of diffusible antifungal metabolites by rhizospheric isolates against *Fusarium oxysporum* f.sp. *vasinfectum*

Sr. No.	Rhizospheric isolates	Growth towards antagonist in mm (R2)	Growth of control in mm (R1)	% of inhibition
1	RLS18	19	40	52.50
2	RLS19	17	40	57.50
3	RLS53	20	40	50.00
4	RLS52	18	40	55.00
5	RLS58	22	40	45.00
6	RLS72	20	40	50.00
7	RLS76	21	40	47.50
8	RLS102	20	40	50.00
9	Control	nil	40	100.00

Table 3: Siderophore production of rhizospheric isolates by modified CAS assay

Sr. No.	Rhizospheric isolate	Siderophore production
1	RLS18	+++
2	RLS19	++
3	RLS53	+++
4	RLS52	++
5	RLS58	+++
6	RLS72	++
7	RLS79	++
8	RLS102	++
9	Control	nil
- = No siderophore production, + = less siderophore production		
++ = less siderophore production +++ = medium siderophore production ++++ = high siderophore production		

uncontaminated soils was studied by using Chrome azurol sulfonate (CAS) was used for both quantitative and qualitative evaluation of siderophores production. The production of siderophores in rhizosphere fungi was higher than those isolated from the contaminated soil (Hussein and Joo, 2012). Siderophore production by Fungal M10 strain was studied using CAS blue agar and the iron(III)-chelating compounds, excreted by the microorganism and diffused through the medium producing a colour change from blue to orange (Vinale *et al.*, 2013). Microbial Siderophores production increases virulence power of microorganisms and serve as good biocontrol agents (Balado *et al.* 2015).

## CONCLUSION

Our results suggest that the rhizospheric isolate RLS19, RLS52 RLS53 and RLS72 inhibits the mycelial growth

of *Fusarium oxysporum* f.sp. *vasinfectum* by producing volatile metabolites diffusible metabolites and siderophore. The cumulative effect of these secondary metabolites resulted in inhibition of mycelial growth of the pathogen causing Fusarium wilt of Bt-cotton and serves as good alternative for chemical control. These four isolate will be serve as excellent microbial control agents against *Fusarium* wilt disease of Bt-cotton and needs to evaluate in field condition.

## ACKNOWLEDGEMENTS

The authors wish to thank Dr. B. R. Chavan Principal, Yogeshwari Mahavidyalaya, Ambajogai for providing facilities to conduct the research.

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