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Screening of antagonistic activity and production of volatile and non- volatile compounds by rhizospheric microbes against *Fusarium oxysporum* f. sp. *cubense in vitro*

Lalngaihawmi and Ashok Bhattacharyya

Abstract
A total of 54 native rhizospheric microbes were screened under *in vitro* for their antagonistic effect against *Fusarium oxysporum* f. sp. *cubense* (Foc), the causal organism of Fusarium wilt of banana. The effect of all the rhizospheric microbes collected during the present investigation significantly differed in terms of inhibition of radial growth of Foc. After 120 hours of incubation, *Trichoderma reesei* (RMF-25) was found most promising as antagonist against Foc with 71.08 per cent inhibition of radial growth followed by *T. reesei* (RMF- 13) with 70.55 per cent and *T. harzianum* (RMF-28) with 70.15 per cent inhibition of radial growth of Foc. The three best promising *Trichoderma* spp. were further screened for their ability to produce secondary metabolites *viz.* volatile and non-volatile compounds. The results revealed that all the *Trichoderma* spp. significantly inhibited the test pathogen by production of toxic metabolites and diffusible non-volatile secondary metabolites. In the case of production f toxic volatile compounds, the per cent inhibition of radial growth of Foc was observed highest by *T. reesei* (RMF 25) with 40.52 per cent inhibition which is closely followed by *T. reesei* (RMF 13) with 40.14 per cent and *T. harzianum* (RMF 28) with 39.03 per cent inhibition. All the *Trichoderma* spp. were also found to produce diffusible non-volatile metabolites where *T. reesei* (RMF- 25) was found to be most promising with 35.96 per cent inhibition of radial growth followed by *T. reesei* (RMF- 13) with 35.22 per cent and *T. harzianum* (RMF- 28) with 34.72 per cent inhibition of radial growth of the test pathogen *in vitro.*

Keywords: Rhizospheric microbes, *in vitro*, Foc, *Trichoderma* spp

Introduction
Fusarium wilt of banana, also known as the Panama disease caused by *Fusarium oxysporum* f. sp *cubense* (Foc), is a historically important disease of bananas worldwide (Ploetz 1990) [19]. Even in India, the disease is widespread in almost all the banana growing states, with disease severity as high as 80-90% in some states where susceptible cultivars are grown in large areas (Mustaffa and Thangavelu, 2011). Foc is one of around 120 formae speciales (special forms) of *F. oxysporum* which cause vascular wilts of flowering plants (Gerlach and Nirenberg, 1982; Minerdi *et al.,* 2008) and generally considered to be one of the most destructive formae speciales of *F. oxysporum* (Ploetz, 1990). Since the first epidemic occurrence of the disease in Panama in 1950s the disease and control methods had been studied (Leong *et al.,* 2009). Due to the perennial nature of bananas and the polycyclic nature of the disease, effective, long-term management of Fusarium wilt remains a challenge and requires the development of new and alternative management strategies (Ghag *et al.,* 2015). The use of biocontrol agents either from the rhizosphere or endophytes has been proved to be an environmental friendly disease management strategy in recent years (Xue *et al.,* 2015, Deltour *et al.,* 2017, Fu *et al.,* 2017) [28, 4, 10]. Bio control agents involve a bewildering array of mechanisms in achieving disease control (Junaid, 2013). Different spp. of *Trichoderma* are able to secrete 40 different secondary metabolites that may contribute to their mycoparasitism and antibiotic action (Meena *et al.,* 2017). Therefore, biological control of Fusarium wilt disease has become an increasingly popular disease management consideration because of its environmental friendly nature which offers a potential alternative to the use of chemical pesticides and the discovery of novel mechanisms of plant protection associated with certain microorganisms (Weller *et al*., 2002; Fravel *et al*., 2003). Thus, understanding the potential of rhizospheric microorganisms in plant disease management, the present work has been undertaken to test the efficacy of native rhizospheric microbes and explore their biocontrol potential against Fusarium wilt of banana *in vitro*.

Materials and methods

The present investigation was carried out in the Department of Plant Pathology, Assam Agricultural University, Jorhat, Assam.

Antagonistic effect of rhizospheric microbes against Foc

Native rhizospheric microbes were isolated and purified from different rhizosphere of banana collected from the state of Assam, Mizoram, Nagaland and Meghalaya. Irrespective of bacterial and fungal isolates, all the rhizospheric microbes were screened under *in vitro* by dual culture method (Dennis and Webster, 1971) against Foc, the causal organism of Fusarium wilt of banana. Culture discs of 5mm diameter each of the fungal rhizospheric micro-organisms and the pathogen were worked out with the help of a sterilized cork borer from the margin of three days old active culture and transferred into PDA medium contained in Petri plates (90mm diameter) on opposite sides approximately at 10mm from the periphery of the plate while rhizospheric bacterial isolates were streaked on the opposite side of the Petri plate (90mm) containing Foc isolates. A control plate having only the test pathogen was also kept for comparison. The Petri plates were then incubated at 25 ±2 º C. The experiment combinations were placed in Completely Randomized Design (CRD) and each combination was replicated three (3) times. Radial growth of the test pathogen, Foc in the presence of rhizospheric microbes was recorded and per cent growth inhibition of the test fungus was calculated Recording of the antagonistic effect was done after every 24 hours up to 120 hours. The respective inhibition was calculated after Vincent (1947) as follows:

$$\text{Per cent inhibition (\%)} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

Identification of rhizospheric microbes

Identification of rhzospheric microbes was carried out only for the three best performing rhizospheric microbes form dual culture plate technique.

Screening of rhizospheric microbes for the production of secondary metabolites

The three best promising rhizospheric microbes were further screened for their ability to produce toxic volatile compounds and diffusible non-volatile compounds.

Volatile compounds production

The three rhizospheric microbes were assayed for their ability to produce volatile compounds following the protocol given by Dennis and Webster (1971) [5]. The rhizospheric microbes were inoculated on the sterile Petri dishes containing PDA. Another plate of same diameter was inoculated with actively growing mycelia discs of Foc pathogen and then inverted over the first plate. The junction of both the petri dishes was sealed tightly with parafilm. PDA medium with Foc at the upper and lower lid was maintained as control. Five replications were maintained for each antagonist and were incubated at 25°C for seven days. The growth of the pathogen after the incubation was measured and per cent inhibition of mycelia growth of the pathogen was calculated according to (Vincent, 1947) [24].

Non-volatile compounds production

For the production of non-volatile compounds by the three effective rhizospheric microorganisms, the protocol given by Dennis and Webster (1971) was followed. The three effective rhizospheric microbes were placed on a sterilized cellophane paper which was placed on top of solidified PDA media containing Petri plates. The rhizospheric microbes were placed separately onto the cellophane paper and incubated at 25±2°C. Control plates were also maintained with cellophane paper only without inoculation of the test organism. Five replications were maintained for each antagonist and were incubated at 25°C for seven days. The plates were then incubated for seven days to check whether the rhizospheric microbes were able to produce diffusible non-volatile compounds. After seven days, careful removal of the cellophane paper along with the organism was done and the same plates were inoculated with Foc for another five days at 25±2°C. The growth of the pathogen after the incubation was measured and per cent inhibition of mycelial growth of the pathogen was calculated according to (Vincent, 1947).

Statistical analysis

The data collected were subjected to statistical analysis by fisher's method of analysis of variance. Significance of variance among the data were calculated out by calculating the 'F' value and comparing it with tabulated value of 'F' at 5 percent level of probability as given by Snedecor and Cochran (1967).

Results

Antagonistic effect of rhizospheric microbes against Foc

A total of 54 native rhizospheric microbes were screened under *in vitro* to test their antagonistic effect against Foc, the causal organism of Fusarium wilt of banana. The effect of all the rhizospheric microbes collected during the present investigation significantly differed in terms of inhibition of radial growth of Foc. The growth of Foc in dual culture plates were observed to progress until they came in contact with the leading edges of the rhizospheric microbes, after which it ceased to grow and only the rhizospheric microbes continued to grow. The per cent inhibition over control was calculated every 24 hours till 120 hours of incubation. The results thus obtained have been presented in Table 1.

At 24 hours of incubation, RMF-25 was found most promising as antagonist against Foc with 33.33 per cent which differed significantly from all other treatments followed by RMF- 28 and RMF-13 with 28.84 per cent inhibition of radial growth. The per cent inhibition recorded by the rest of the rhizospheric microbes against Foc at 24 hours incubation ranged from 18.58 per cent in case of RMB-2 to 28.20 per cent in case of RMF- 3, RMF- 8 and RMF- 32.

At 48 hours of incubation, the highest inhibition was recorded with RMF- 25 which differed significantly from all the other treatments with 58.49 per cent inhibition followed by RMF-13 (55.88%) and RMF- 28 (55.22%). The per cent inhibition recorded by the rest of the rhizospheric microbes ranged from 46.07 per cent to 54.90 per cent. Per cent inhibition of radial growth of Foc by RM- 25 *in vitro* significantly differed from all other treatments. However, inhibition of radial growth of Foc by rhizospheric microbes like RMF- 13, RMF- 28, RMF-32, RMF- 35, RMF- 39 were found to be statistically *at par*.

Similar observations were recorded at 72, 96 and 120 hours of incubation with rhizospheric microbe RMF-25 with 59.95, 67.72 and 71.08 per cent inhibition respectively. The second highest per cent inhibition was by rhizospheric microbe RMF-13 with 57.97, 66.45 and 70.55 per cent at 72, 96 and 12 hours respectively. The third highest per cent inhibition over control was recorded by rhizospheric microbe RMF-28 with

57.49, 65.66 and 70.15 per cent at 72, 96 and 12 hours respectively. However, at 120 hours of incubation, the inhibition of radial growth of Foc by rhizospheric microbes RMF- 13 and RMF- 28 were found to be statistically *at par*.

From the overall results of the antagonistic activities of rhizospheric microbes against Foc, fifteen rhizospheric microbes having the capability of inhibiting more than 65 per cent of the test pathogen were considered to be effective against Foc, the causal organism of Fuarium wilt of banana. These include RMF- 3, RMF- 8, RMF- 9, RMF- 13, RMF- 14, RMF- 17, RMF- 18, RMF- 23, RMF- 24, RMF- 25, RMF- 28, RMF- 31, RMF- 32, RMF- 35 and RMF- 39. The antagonistic effects of these promising rhizospheric microbes have been presented in Plate 1.

Table 1. Antagonistic effect of rhizospheric microbes against Foc by dual culture technique

Treatment	Growth of Foc (cm)	PI* of Foc in presence of RMs	Growth of Foc (cm)	PI of Foc in presence of RMs	Growth of Foc (cm)	PI of Foc in presence of RMs	Growth of Foc (cm)	PI of Foc in presence of RMs	Growth of Foc (cm)	PI of Foc in presence of RMs
	24 hrs		48 hrs		72 hrs		96 hrs		120 hrs	
RMF* 1+Foc	1.20	23.07	1.50	50.98	2.32	48.09	3.37	46.67	4.30	42.97
RMF 2+Foc	1.14	26.92	1.59	48.03	2.36	47.20	3.44	45.56	4.39	41.77
RMF 3+Foc	1.12	28.20	1.45	52.61	2.08	53.46	2.33	63.13	2.40	68.16
RMF 4+Foc	1.17	25.00	1.48	51.63	2.30	48.54	3.38	46.51	4.23	43.89
RMF 5+Foc	1.18	24.35	1.53	50.00	2.35	47.42	3.45	45.41	3.69	51.06
RMF 6+Foc	1.19	23.71	1.50	50.98	2.41	46.08	2.41	61.86	3.76	50.13
RMF 7+Foc	1.17	25.00	1.47	51.96	2.27	49.21	3.57	43.51	3.57	52.65
RMF 8+Foc	1.12	28.20	1.44	52.94	2.01	55.03	2.30	63.60	2.36	68.70
RMF 9+Foc	1.16	25.64	1.39	54.57	2.03	54.58	2.28	63.92	2.34	68.96
RMF 10+Foc	1.19	23.71	1.54	49.67	2.30	48.54	3.55	43.82	4.31	42.83
RMF 11+Foc	1.21	22.43	1.61	47.38	2.38	46.75	3.53	44.14	4.59	39.12
RMF 12+Foc	1.20	23.07	1.56	49.01	2.42	45.86	3.47	45.09	3.82	49.33
RMF 13+Foc	1.11	28.84	1.35	55.88	1.88	57.94	2.12	66.45	2.22	70.55
RMF 14+Foc	1.15	26.28	1.45	52.61	2.05	54.13	2.20	65.18	2.38	68.43
RMF 15+Foc	1.22	27.86	1.59	48.03	2.30	48.54	3.27	48.25	3.52	53.31
RMF 16+Foc	1.24	20.51	1.56	49.01	2.31	48.32	3.32	47.46	3.59	52.38
RMF 17+Foc	1.15	26.28	1.41	53.92	2.09	53.24	2.29	63.76	2.37	68.56
RMF 18+Foc	1.14	26.92	1.39	54.57	2.04	54.36	2.33	63.13	2.38	68.43
RMF 19+Foc	1.21	22.43	1.52	50.32	2.32	48.09	3.39	46.36	3.46	54.11
RMF 20+Foc	1.22	27.86	1.56	49.01	2.36	47.20	3.40	46.20	3.51	53.44
RMF 21+Foc	1.24	20.51	1.61	47.38	2.39	46.53	3.43	45.72	3.96	47.48
RMF 22+Foc	1.21	22.43	1.65	46.07	2.41	46.08	3.45	45.41	3.70	50.92
RMF 23+Foc	1.14	26.92	1.42	53.59	2.09	53.24	2.23	64.71	2.46	67.37
RMF 24+Foc	1.13	27.56	1.39	54.57	2.00	55.25	2.20	65.18	2.30	69.49
RMF 25+Foc	1.04	33.33	1.27	58.49	1.79	59.95	2.04	67.72	2.18	71.08
RMF 26+Foc	1.22	27.86	1.50	50.98	2.36	47.20	3.43	45.72	3.65	51.59
RMF 27+Foc	1.24	20.51	1.45	52.61	2.40	46.30	3.48	44.93	4.20	44.29
RMF 28+Foc	1.11	28.84	1.37	55.22	1.90	57.49	2.17	65.66	2.25	70.15
RMF 29+Foc	1.24	20.51	1.62	47.05	2.40	46.30	3.42	45.88	3.72	50.66
RMF 30+Foc	1.19	23.71	1.60	47.71	2.35	47.42	3.33	47.31	3.99	47.08
RMF 31+Foc	1.14	26.92	1.39	54.57	2.11	52.79	2.26	64.24	2.43	67.77
RMF 32+Foc	1.12	28.20	1.38	54.90	2.14	52.12	2.27	64.08	2.44	67.63
RMF 33+Foc	1.25	19.87	1.62	47.05	2.41	46.08	3.47	45.09	3.88	48.54
RMF 34+Foc	1.26	19.23	1.61	47.38	2.42	45.86	3.57	43.51	4.33	42.57
RMF 35+Foc	1.13	27.56	1.38	54.90	2.17	51.45	2.28	63.92	2.37	68.56
RMF 36+Foc	1.25	19.87	1.59	48.03	2.36	47.20	3.46	45.25	3.78	49.86
RMF 37+Foc	1.26	19.23	1.60	47.71	2.41	46.08	3.48	44.93	3.79	49.73
RMF 38+Foc	1.25	19.87	1.59	48.03	2.34	47.65	3.46	45.25	3.61	52.12
RMF 39+Foc	1.15	26.28	1.38	54.90	1.99	55.48	2.25	64.39	2.42	67.90
RMF 40+Foc	1.24	20.51	1.65	46.07	2.37	46.97	3.47	45.09	3.81	49.46
RMF 41+Foc	1.25	19.87	1.55	49.34	2.35	47.42	3.45	45.41	3.91	48.14
RMB* 1+Foc	1.19	23.71	1.53	50.00	2.36	47.20	3.51	44.46	4.01	46.81
RMB 2+Foc	1.27	18.58	1.58	48.36	2.37	46.97	3.53	44.14	4.22	44.03
RMB 3+Foc	1.24	20.51	1.64	46.40	2.38	46.75	3.56	43.67	3.95	47.61
RMB 4+Foc	1.25	19.87	1.65	46.07	2.35	47.42	3.58	43.35	4.19	44.42
RMB 5+Foc	1.20	23.07	1.62	47.05	2.40	46.30	3.51	44.46	4.04	46.41
RMB 6+Foc	1.21	22.43	1.66	45.75	2.41	46.08	3.54	43.98	3.93	47.87
RMB 7+Foc	1.25	19.87	1.62	47.05	2.42	45.86	3.52	44.30	3.96	47.48
RMB 8+Foc	1.23	21.15	1.63	46.73	2.39	46.53	3.55	43.82	4.07	46.02
RMB 9+Foc	1.19	23.71	1.64	46.40	2.41	46.08	3.54	43.98	4.19	44.42
RMB 10+Foc	1.20	23.07	1.64	46.40	2.42	45.86	3.57	43.51	4.14	45.09
RMB 11+Foc	1.25	19.87	1.63	46.73	2.40	46.30	3.50	44.62	4.27	43.36
RMB 12+Foc	1.26	19.23	1.64	46.40	2.42	45.86	3.57	43.51	4.37	42.02
RMB 13+Foc	1.23	21.15	1.65	46.07	2.41	46.08	3.54	43.98	4.35	42.04
Control	1.56	00.00	3.06	00.00	4.47	00.00	6.32	00.00	7.54	00.00
SE.d±	0.020		0.038		0.096		0.030		0.058	
CD (p=0.05)	0.040		0.075		0.190		0.059		0.114	

PI = Per cent inhibition, RMF = Rhizospheric Microbes- Fung, RMB= Rhizospheric microbes- Bacteria

Identification of rhizospheric microbes

The three best promising rhizospheric microbes *viz*. RMF-25, RMF-13 and RMF-28 were identified by sequencing of 18s rRNA and were identified as *Trichoderma reesei* (RMF-25 and RMF-13) and *T. harzianum* (RMF-28).

Volatile compounds production

The study on the volatile compounds production potentiality of the three promising rhizospheric microbes revealed that volatile compound production by the rhizospheric microbes were inversely proportional the growth of Foc and directly proportional to per cent inhibition of Foc. All the *Trichoderma* spp. significantly inhibited the test pathogen by production of toxic volatile compounds. The results thus obtained have been presented in Table 2. and depicted in Plate 2. The per cent inhibition of radial growth of Foc was observed highest by RMF 25 (40.52 %) that is closely followed by RMF 13 (40.14 %) and RMF 28 (39.03 %). However inhibition of radial growth of all the three rhzizospheric microbes was found to be statistically *at par.*

Table 2: Volatile compounds production test of three promising *Trichoderma* spp.

S. No.	Rhizospheric Microbes	Growth (cm) of Foc at 7 DAI	Per cent inhibition of Foc at 7 DAI
1.	*T. reesei* (RMF-25)	3.20	40.52
2.	*T. reesei* (RMF-13)	3.22	40.14
3.	*T. harzianum* (RMF-28)	3.28	39.03
4.	Control	5.38	0.00
	SEd±	0.15	
	CD (p=0.05)	0.06	

DAI= Days after inoculation

Non-volatile compounds production

The study on the non-volatile compounds production potentiality of the three promising rhizospheric microbes also revealed that non-volatile compound produced by the three promising rhizospheric microbes were inversely proportional the growth of Foc and directly proportional to per cent inhibition of Foc. All the *Trichoderma* spp. produced non-volatile compounds having significant effect in reducing the radial growth of test pathogen. The results thus obtained have been presented in Table 3 and depicted in Plate 3. Among the three *Trichoderma* spp., RMF 25 was found most promising in producing non-volatile compounds against Foc with 35.96 per cent inhibition of radial growth followed by RMF 13 (35.22 %) and RMF 28 (34.72 %). Per cent inhibition of radial growth of RMF 25 significantly differed from the other two rhizospheric microbes while RMF 13 and RMF 25 were found to be statistically *at par*.

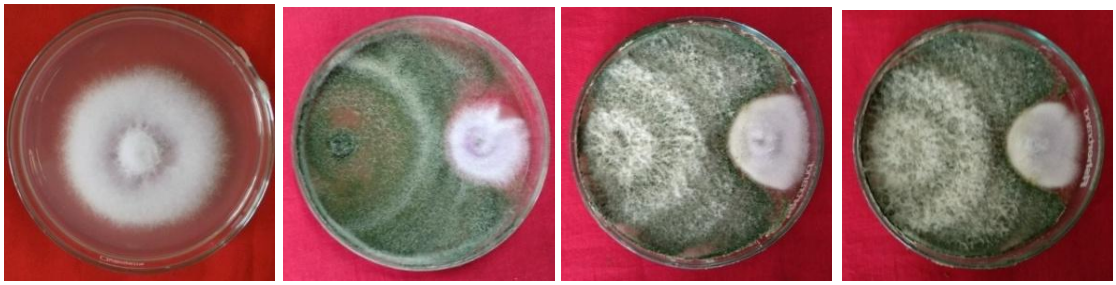
Table: Non-volatile production test for three promising *Trichoderma* spp.

S. No.	Rhizospheric Microbes	Growth (cm) of Foc 7 DAI	Per cent inhibition of Foc 7 DAI
1.	*T. reesei* (RMF-25)	5.20	35.96
2.	*T. reesei* (RMF-13)	5.26	35.22
3.	*T. harzianum* (RMF-28)	5.30	34.72
4.	Control	8.12	0.00
	SEd±	0.05	
	CD (p=0.05)	0.10	

DAI= Days after inoculation

Discussions

In the present investigation concerning the inhibitory effect of native rhizospheric microbes against Fusarium wilt caused by *Fusarium oxysporum* f.sp. *cubens*, most of the rhizospheric microbes were reported to have antagonistic effect on the pathogen *in vitro*, though at different levels. It is evident from earlier works that rhizospheric microbes have antagonistic effect against Foc (Akila *et al*., 2011; Thangavelu and Gopi, 2015; Baruah *et al*, 2018) [3] who reported that native rhizospheric microbes collected from different banana rhizosphere could inhibit the growth of Foc either singly or in combination. Several reports have also revealed that different species of *Trichoderma* possess the ability to control different phytopathogenic diseases (Elad *et al*., 1998; Elad and Kapat 1999; Xu *et al*., 1999; Abdel-Fattah *et al*., 2007, Ru and Di, 2012). The inhibition of the pathogen by *Trichoderma* spp. was mainly due different modes of action of *Trichoderma* spp. including production of different antifungal compounds like volatile and non-volatile secondary metabolites (Dubey *et al*., 2007, Waseem *et al*., 2013; Thangavelu and Gopi, 2015; Nagamani *et al*., 2017) who also reported the production of these compounds in inhibiting certain pathogens. Since the data obtained from the present investigation also indicates significant reduction in the growth of Foc as well as inhibition of the pathogen by the production of volatile and non-volatile secondary metabolites, thus it corroborates with the findings of the earlier workers.



a) CONTROL **b) RMF 3 + Foc** **c) RMF 8 + Foc** **d) RMF 9 + Foc**

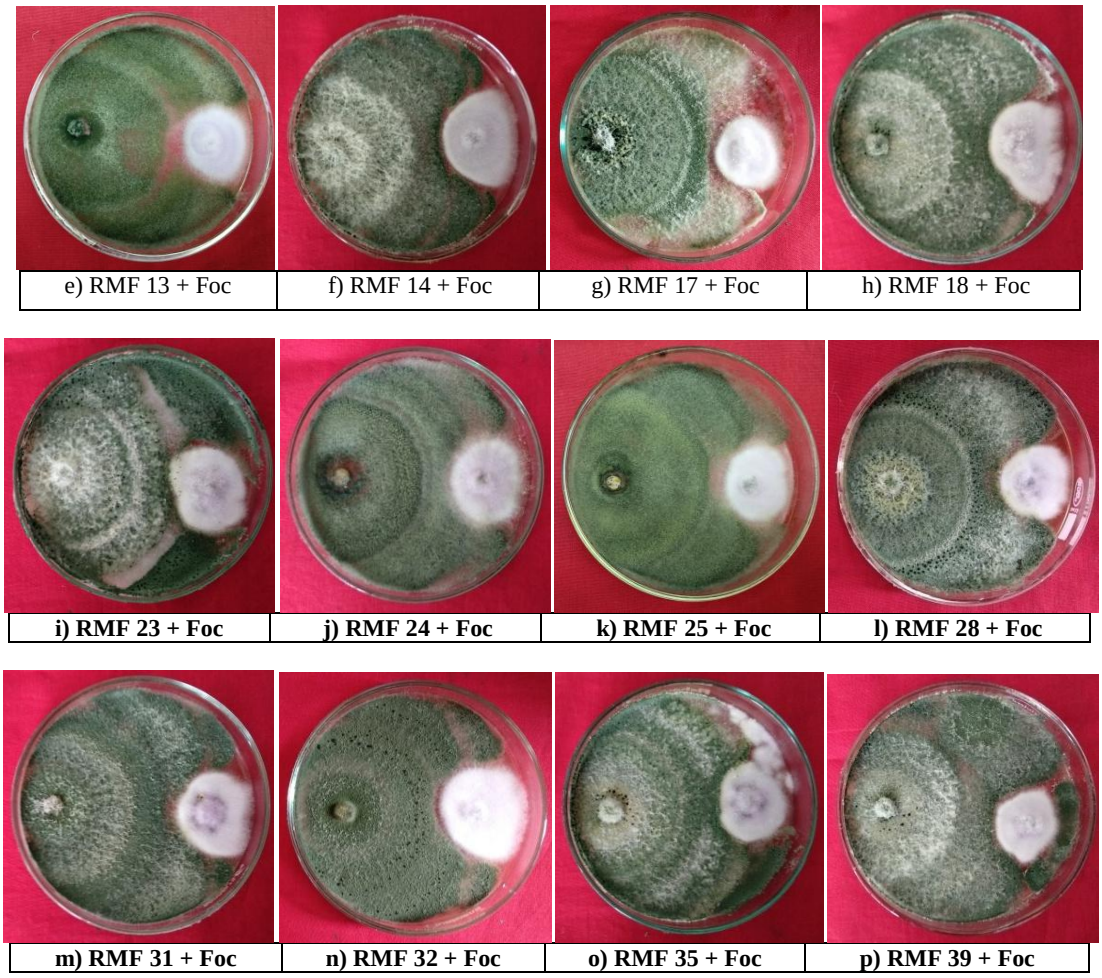


Plate 1: Antagonistic effect of fifteen effective rhizospheric microbes against Foc after 120 hours of inoculation

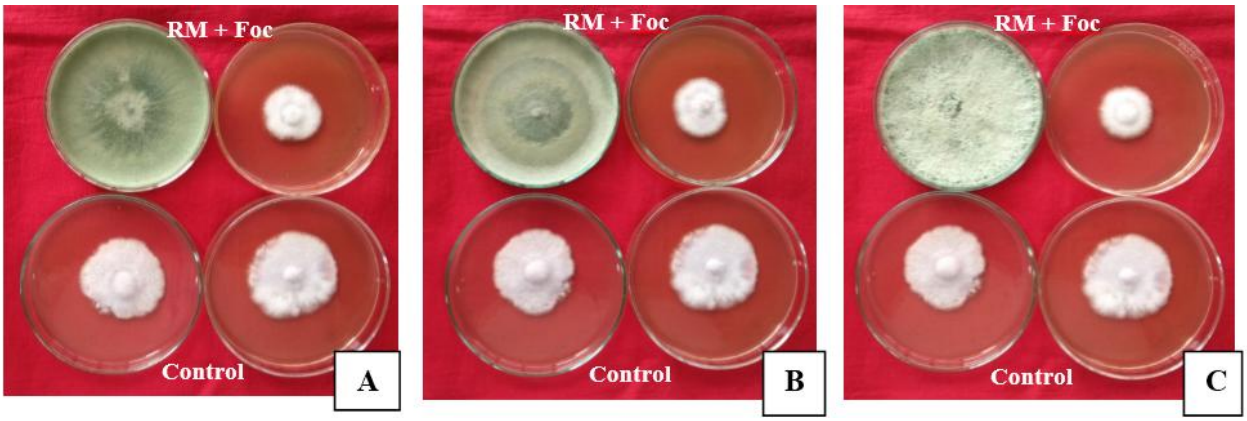


Plate 2. Volatile compounds production assay by promising rhizospheric microbes

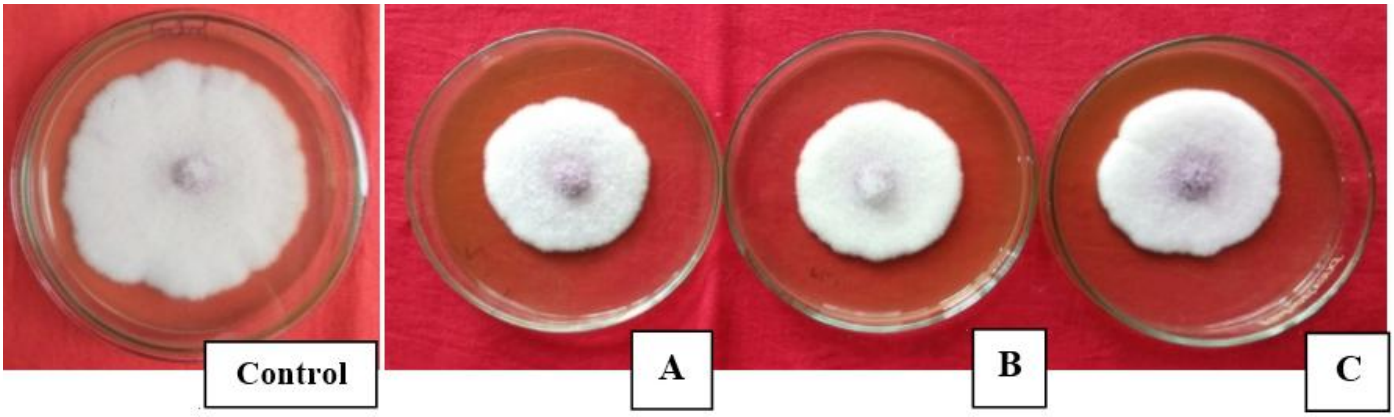


Plate 3 Non-volatile compounds production assay by promising rhizospheric microbes A) B) C)

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