

## Evaluation of antimicrobial and antagonistic activities of endophytic bacterial isolates of an ethnomedicinal plant of Manipur, Shamba (*Oroxylum indicum*)

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### ABSTRACT

In the present study, a total of 57 endophytic bacterial isolates were obtained from leaf, stem and root samples of *Oroxylum indicum* using Nutrient Agar (NA) and Starch Casein Nitrate Agar (SCNA). The isolates were screened for antibacterial activity against a panel of test bacterial pathogens viz. *Micrococcus luteus* (MTCC 106), *Bacillus subtilis* (MTCC 121) and *Escherichia coli* (MTCC 739) and also tested for antagonistic activity against fungal pathogen viz. *Fusarium oxysporium* (MTCC 287). Among 57 isolates, 5 isolates (OInL1, OInL6, OInL7, OInL11 and OInL14), 12 isolates (OInL12, OInL13, OInS1, OInS2, OInS13, OInS15, OInS19, OInS20, OInR8, OInR12, OInR13 and OInR15) and 10 isolates (OInL1, OInL4, OInL7, OInL12, OInS15, OInS20, OInR13, OInR15, OInR16 and OInR17) exhibited antibacterial activity against *Micrococcus luteus* (MTCC 106), *Bacillus subtilis* (MTCC 121) and *Escherichia coli* (MTCC 739) respectively in secondary antimicrobial screening using Kirby Bauer method. Furthermore, among 57 isolates, 9 (OInL6, OInL7, OInL11, OInL12, OInL14, OInR13, OInR15, OInR16 and OInR17) showed antagonistic activity against *Fusarium oxysporium* (MTCC 287).

Figures : 06

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KEY WORDS : Antibacterial activity, Antifungal activity, Endophytic bacteria, *Oroxylum indicum*

### Introduction

Endophytes are microorganisms that live inside living plant tissues without causing any negative effect to host plants.<sup>7</sup> Endophytes are known as prolific producers of natural products with antibacterial, antifungal, anticancer, immunostimulatory, antibiotics, bioinoculants, and agroactive compounds etc.<sup>9</sup> With increase in the number of drug resistant super bugs and opportunistic fungal infections, causing threats to crops yields, there is an urgent requirement for new drugs, antibiotics or biocontrol agents to combat these diseases.

Of late, several novel antibiotics have been discovered from endophytic actinobacteria e.g. munumbicins, kakadumycin and coronamycin.<sup>3-5</sup> These compounds were obtained from *Streptomyces* spp. endophytic in *Kennedia nigriscans*, *Grevillea pteridifolia* and *Monstera* sp. and they exhibited remarkable

antibacterial, antifungal and antimalarial activities. Manipur, a north eastern states of India is home to many endemic flora and fauna and is located between 23.830N and 25.680N latitude and 93.030 E and 94.780 E longitudes.<sup>1</sup> It holds special promise for bioprospecting of endophytic bacteria of medicinal and agricultural importance as it falls under Indo-Burma biodiversity hotspot.

*Oroxylum indicum* is an endangered rare medicinal plant used in the treatments of many ailments in ayurvedic, herbal and folk medicine. Each plant part possessed medicinal values such as antimicrobial, antifungal, antioxidant, anti-inflammatory, anticancer etc.<sup>9</sup> In this study, endophytic bacterial isolates were isolated from leaves, stems and roots samples of *Oroxylum indicum* and screened for its antimicrobial activity by primary antimicrobial screening via cross streak and secondary antimicrobial screening by Kirby

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TABLE-1 : Secondary screening by Kirby Bauer method

S.No.	Isolate No.	<i>Micrococcus luteus</i> MTCC 106	<i>Bacillus subtilis</i> MTCC 121	<i>Escherichia coli</i> MTCC 739
		Inhibition Zone (mm)		
1	OInL1	10 mm	–	12 mm
2	OInL2	–	–	–
3	OInL3	–	–	–
4	OInL4	–	–	8 mm
5	OInL5	–	–	–
6	OInL6	11 mm	–	–
7	OInL7	10 mm	–	11 mm
8	OInL8	–	–	–
9	OInL9	–	–	–
10	OInL10	–	–	–
11	OInL11	11 mm	–	–
12	OInL12	–	6 mm	9 mm
13	OInL13	–	9 mm	–
14	OInL14	11 mm	–	–
15	OInL15	–	–	–
16	OInS1	–	13 mm	–
17	OInS2	–	15 mm	–
18	OInS3	–	–	–
19	OInS4	–	–	–
20	OInS5	–	–	–
21	OInS6	–	–	–

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22	OlnS7	-	-	-
23	OlnS8	-	-	-
24	OlnS9	-	-	-
25	OlnS10	-	-	-
26	OlnS11	-	-	-
27	OlnS12	-	-	-
28	OlnS13	-	8 mm	-
29	OlnS14	-	-	-
30	OlnS15	-	16 mm	7 mm
31	OlnS16	-	-	-
32	OlnS17	-	-	-
33	OlnS18	-	-	-
34	OlnS19	-	7 mm	-
35	OlnS20	-	10 mm	13 mm
36	OlnR1	-	-	-
37	OlnR2	-	-	-
38	OlnR3	-	-	-
39	OlnR4	-	-	-
40	OlnR5	-	-	-
41	OlnR6	-	-	-
42	OlnR7	-	-	-
43	OlnR8	-	15 mm	-
44	OlnR 9	-	-	-
45	OlnR10	-	-	-

46	OInR11	–	–	–
47	OInR12	–	7 mm	–
48	OInR13	–	6 mm	12 mm
49	OInR14	–	–	–
50	OInR15	–	8 mm	12 mm
51	OInR16	–	–	13 mm
52	OInR17	–	–	10 mm
53	OInR18	–	–	–
54	OInR19	–	–	–
55	OInR20	–	–	–
56	OInR21	–	–	–
57	OInR22	–	–	–

assay against three test pathogens<sup>2</sup>; *Micrococcus luteus* (MTCC 106), *Bacillus subtilis* (MTCC 121) and *Escherichia coli* (MTCC 739). All 57 isolates were also further screened for antifungal activity against *Fusarium oxysporum* (MTCC 287) using Dual culture method.

## Materials and Methods

### Isolation of endophytic bacteria

The leaf, stem and root samples of the ethnomedicinal plant of Manipur, *Oroxylum indicum* were collected in November 2018 from Khurkhul, Imphal west, Manipur (24.93°N, 93.87°E). The samples were washed thoroughly under running tap water and surface sterilized by sequential treatment with the following solutions: 4-10 min wash in 5% sodium hypochlorite, 10 min wash in 2.5% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 5 min wash in 75% Ethyl alcohol followed by washing in distilled water and a final rinse in 10% NaHCO<sub>3</sub>.<sup>8</sup> Isolation was done on different media viz. NA and SCNA. Efficiency of surface sterilization procedure was checked by culturing aliquots of water from the last rinsing onto the same culture media. Colonies with bacteria like morphologies were picked up and sub-cultured to obtain pure cultures. The purified cultures were preserved as glycerol stocks (20% v/v, -20 °C) for further use.

### Test organisms

Human pathogens viz. *Micrococcus luteus* (MTCC 106), *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 739) and fungal pathogen, *Fusarium oxysporum* (MTCC 287) were procured from Microbial type Culture collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The strains were grown and maintained on Nutrient agar media.

### Primary antibacterial screening:

Primary antibacterial screening was carried out using CrossStreak Method against the test pathogens viz. *Micrococcus luteus* (MTCC 106), *Bacillus subtilis* (MTCC 121) and *Escherichia coli* (MTCC 739). The bacterial isolates were streaked in the middle of the NA plates and kept incubated at 30°C for 2-3 days. Then the freshly grown test pathogens (MTCC 106, MTCC 121 and MTCC 739) were streaked perpendicular to the bacterial isolates and incubated for another 48 hrs. Zone of growth inhibition were then measured.

### Secondary screening

The bacterial isolates were screened for secondary antibacterial activity against the test pathogens viz. *Micrococcus luteus* (MTCC 106), *Bacillus subtilis* (MTCC 121) and *Escherichia coli*<sup>2</sup> (MTCC 739).

**TABLE-2: Biocontrol assay by Dual Culture Method**

S. No.	Bacterial isolates.	% growth inhibition $= \frac{R1 - R2}{R1} \times 100$
1	OInL1	–
2	OInL2	–
3	OInL3	–
4	OInL4	–
5	OInL5	–
6	OInL6	61%
7	OInL7	38%
8	OInL8	–
9	OInL9	–
10	OInL10	–
11	OInL11	61%
12	OInL12	65%
13	OInL13	–
14	OInL14	77%
15	OInL15	–
16	OInS1	–
17	OInS2	–
18	OInS3	–
19	OInS4	–
20	OInS5	–
21	OInS6	–

22	OInS7	–
23	OInS8	–
24	OInS9	–
25	OInS10	–
26	OInS11	–
27	OInS12	–
28	OInS13	–
29	OInS14	–
30	OInS15	–
31	OInS16	–
32	OInS17	–
33	OInS18	–
34	OInS19	–
35	OInS20	–
36	OInR1	–
37	OInR2	–
38	OInR3	–
39	OInR4	–
40	OInR5	–
41	OInR6	–
42	OInR7	–
43	OInR8	–
44	OInR9	–
45	OInR10	–

46	OInR11	–
47	OInR12	–
48	OInR13	57%
49	OInR14	–
50	OInR15	42%
51	OInR16	56%
52	OInR17	61%
53	OInR18	–
54	OInR19	–
55	OInR20	–
56	OInR21	–
57	OInR22	–

The Bacterial isolates and test pathogens were inoculated in Nutrient broth (NB) and incubated at 30°C in an orbital shaker (150 rpm, 7 days). The fully grown bacterial cultures were centrifuged at 10,000 rpm for 15 min and culture supernatant collected in sterile eppendorf tubes. 100 µl of freshly grown test pathogens were spread plated on NA plate, pH 7 and left for drying. Agar wells (6 mm diameter) were punched onto the dried plates. The culture supernatants of bacterial isolates were then put into the wells and plates were incubated

at 30°C for 2-4 days. Presence or absence of inhibition zone around the wells was then checked for growth inhibition.

### Biocontrol assay against fungal pathogen (Dual Culture Method)

The bacterial isolates were screened for antifungal activity against *Fusarium oxysporum* (MTCC 287), by **Dual Culture Method**.<sup>6</sup> This test was done on PDA plates. Agar wells (6 mm diameter) were made on PDA plates using sterilized cork borer and agar plugs from the freshly grown endophytic bacterial isolates were placed on PDA wells in duplicate. The plates were incubated at 30°C for 48 hr. Then agar plugs of the test fungal pathogens were placed at the centres of labelled plates. A plate containing a fungal agar plug without any isolate plug was kept as control. All plates were incubated at 30°C until the control plate shows full growth.

The Percentage growth inhibition was calculated using the following formula:

$$\text{Growth inhibition} = \frac{R1 - R2}{R1} \times 100$$

Radius of bacterial growth in test plate

## Results and Discussion

### Isolation of endophytic bacteria from *Oroxylum indicum*:

A total of 57 bacterial isolates were obtained from leaf, stem and root samples of ethnomedicinal plant *Oroxylum indicum* using two different media viz. NA and SCNA. **Figure1**. Shows mother plates from which we have isolated the pure colonies of bacterial isolates.

15 isolates were obtained from leaf samples, labelled as OInL1-OInL15, 20 from stem (OInS1-

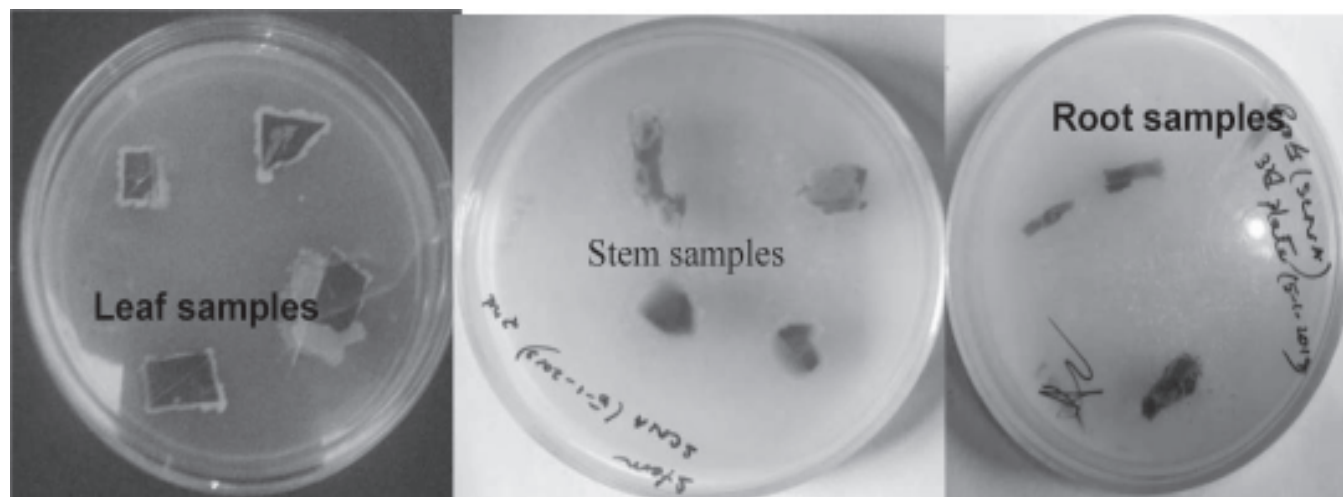
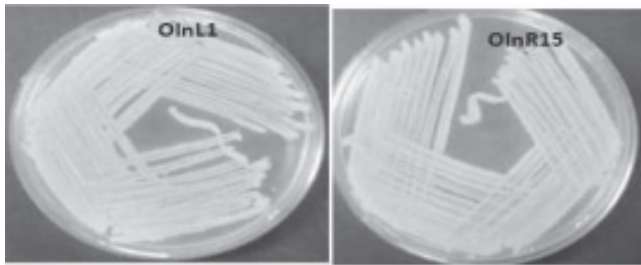
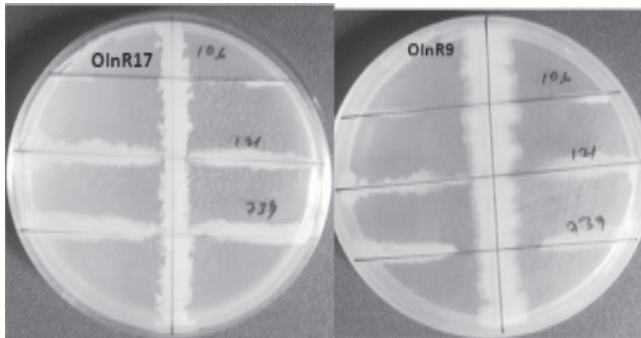


Fig.1: Mother plates of leaf, stem and root samples of *Oroxylum indicum*



**Fig. 2: Representative Plates showing pure cultures of bacterial endophytes isolates from *Oroxylum indicum***

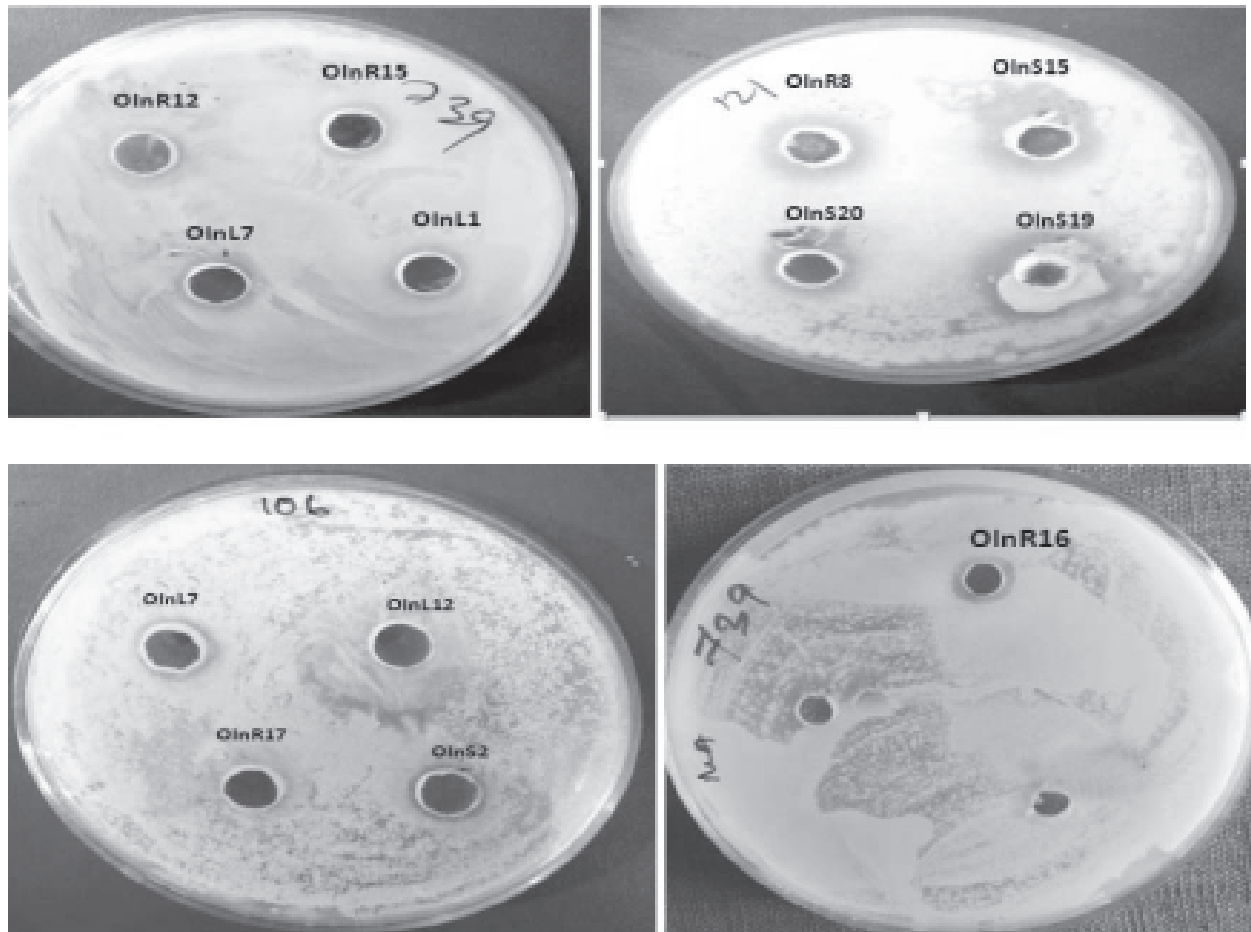


**Fig. 3: Primary antibacterial screening by cross streak method**

OlnS20) and 22(OlnR1-OlnR22) from root using two different media, NA and SCNA.

**Primary Antibacterial screening:**

All 57 isolates were subjected to primary antimicrobial activity against a panel of test pathogens. The isolates were subjected to primary antimicrobial activity against *Micrococcus luteus* (MTCC 106), *Bacillus subtilis* (MTCC 121) and *Escherichia coli* (MTCC 739). 4 isolates (OlnR9, OlnR12, OlnS8 and OlnS20) showed antibacterial activity against all the three test pathogens. 21 isolates (OlnL1, OlnL4, OlnL6, OlnL7, OlnL11, OlnL12, OlnS2, OlnS4, OlnS6, OlnS8, OlnS15, OlnS19, OlnS20, OlnR5, OlnR9, OlnR10, OlnR12, OlnR13, OlnR15, OlnR16 and OlnR17), 7 (OlnS1, OlnS5, OlnS8, OlnS13, OlnS20, OlnR9 and OlnR10) and 13 isolates (OlnL6, OlnS2, OlnS5, OlnS7, OlnS8, OlnS10, OlnS11, OlnS13, OlnS20, OlnR9, OlnR10, OlnR13 and OlnR18) exhibited antibacterial activity against *Micrococcus luteus* (MTCC 106), *Bacillus subtilis* (MTCC 121) and *Escherichia coli* (MTCC 739) respectively in primary antibacterial activity by cross Streak Method (**Figure3**).



**Fig. 4: Secondary screening by Kirby Bauer Method**

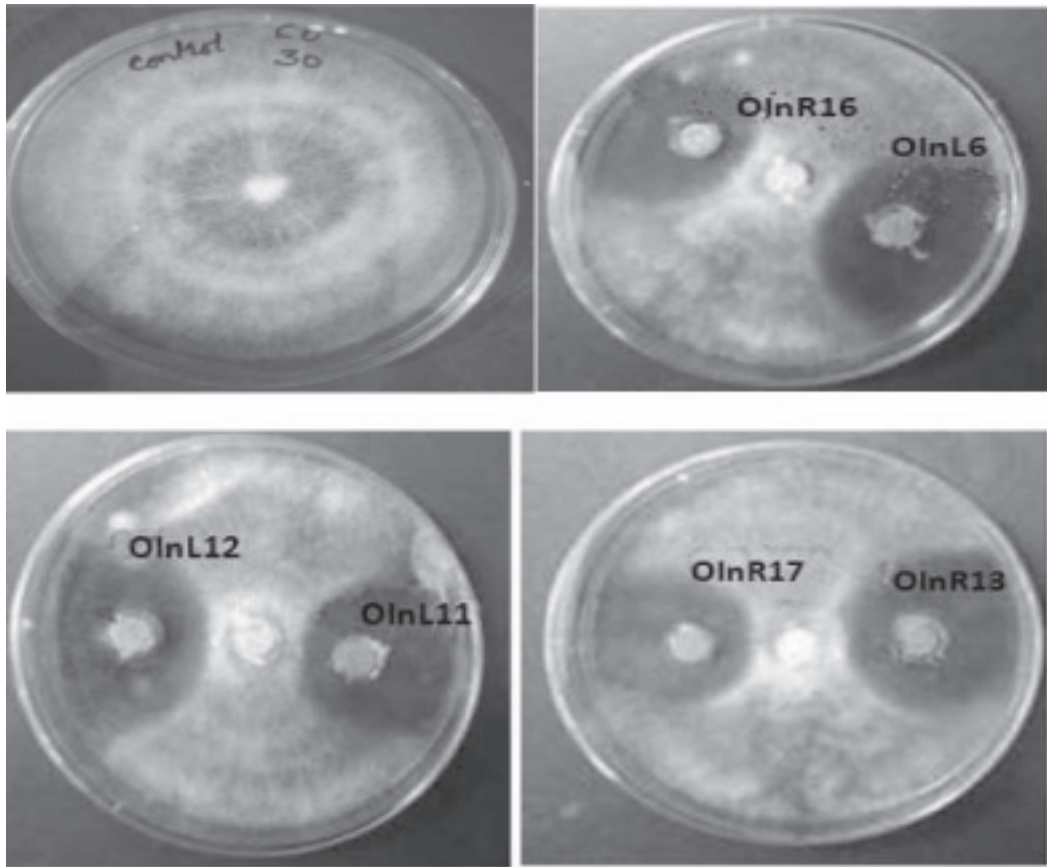


Fig. 5: Biocontrol assay by Dual Culture Method

**Secondary antibacterial activity:**

All 57 bacterial isolates were subjected to secondary antimicrobial screening against three test pathogens; *Micrococcus luteus* (MTCC 106), *Bacillus subtilis* (MTCC 121) and *Escherichia coli* (MTCC 739)

by Kirby-Bauer method. 5 isolates (OInL1, OInL6, OInL7, OInL11 and OInL14) and 12 (OInL12, OInL13, OInS1, OInS2, OInS13, OInS15, OInS19, OInS20, OInR8, OInR12, OInR13 and OInR15) exhibited antibacterial activity against *Micrococcus luteus* (MTCC 106) and

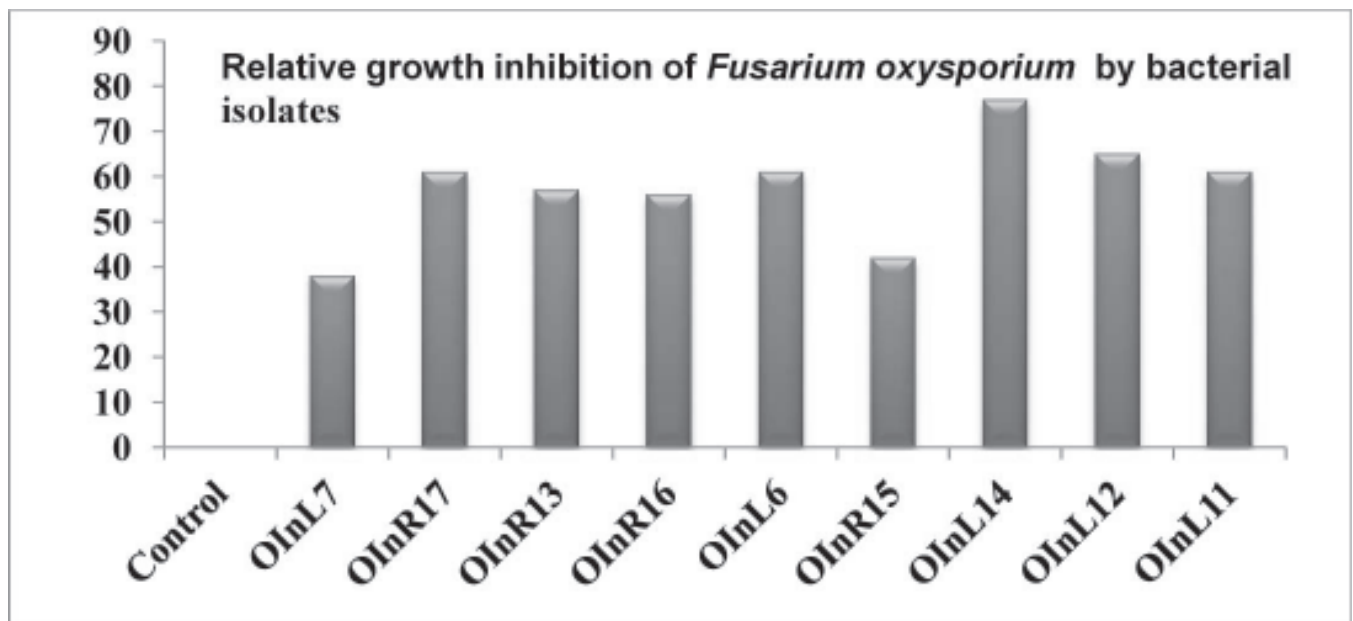


Fig. 6: Relative percentage growth inhibition of *Fusarium oxysporium* by the bacterial isolates



*Bacillus subtilis* (MTCC 121) respectively, whereas 10 isolates (OlnL1, OlnL4, OlnL7, OlnL12, OlnS15, OlnS20, OlnR13, OlnR15, OlnR16 and OlnR17) inhibited *Escherichia coli* (MTCC 739) (Fig. 4 and Table-1).

### Antifungal activity

All 57 isolates were screened for antifungal activity against *Fusarium oxysporum* (MTCC 287) using **Dual Culture Method**. 9 isolates (OlnL6, OlnL7, OlnL11, OlnL12, OlnL14, OlnR13, OlnR15, OlnR16 and OlnR17) exhibited antifungal activity against *Fusarium oxysporum* (MTCC 287) (Fig. 5 and Table-2).

The percentage of growth inhibition was calculated using the following formula:

**Percentage of growth inhibition** =  $((R1-R2)/R1) \times 100$

Where, R1 represents the radial growth (mm) of the test pathogen in the control plate and R2 is the radial growth (mm) of the bacterial isolates in the test plate.

The percentage of growth inhibition by bacterial

isolates is shown in Fig. 6.

Among 57 isolates, 5 isolates (OlnL14, OlnL12, OlnL6, OlnL11 and OlnR17) have exhibited significant antifungal activity and holds promise for further development as bio-control agent for rice cultivation.

### Conclusion

A total of 57 bacterial isolates were obtained using two media, NA and SCNA. Among 57 isolates, 22 isolates exhibited antibacterial activity against one or more test pathogens in primary antibacterial screening while 20 isolates showed antibacterial activity against one or two test pathogens in secondary antibacterial screening by Kirby Bauer method. Moreover, 9 isolates had showed antagonistic activity against *Fusarium oxysporum*. Among 9 isolates, OlnL14, OlnL12, OlnL6, OlnL11 and OlnR17 exhibited significant activity and hold promise for further development as biocontrol agent for rice cultivation.

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