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Preventive measures against contamination of vegetables by endophytic *Salmonella sp*. with antibacterial property of *Ocimum basilicum L*.

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ABSTRACT

Spinach samples (n=120) were collected from various zones of Surat city, Gujarat from December 2018 to November 2019. The accumulated samples were investigated (ISO 6579-1:2017 E) for endophytic contamination of Salmonella sp. From 120 collected samples 16 samples (% prevalence=13.3) were found positive for the endophytic Salmonella sp. which is a significant sign of vegetable contamination with this pathogenic bacteria. Lab-scale, pot, and field experiments were conducted on soil treatment from January 2018 to November 2020 to study the antibacterial effect of Ocimum basilicum (OB) using different concentrations of methanolic OB extracts(3.90,7.81,15.62,31.25, 62.5, 125, 250, and 500 µl /mL) with spinach as test crop. The experiment was constructed in an absolute randomized design with three repetitions. Results revealed that the plant was prevented from endophytic Salmonella sp. contamination on the treatment of soil by application of MIC concentration of OB extract, which was determined 125 mL L^{-1} kg^{-1}. Application of OB extract had significantly prevented the contamination of endophytic Salmonella sp. Growth curve studies of endophytic Salmonella isolates were carried out with treated and untreated soil, which showed significant control and prevention of Salmonella sp. on treatment with OB extracts at MIC. Thus, results suggested that application of MIC 125 mL L^{-1} kg^{-1} of OB extract proved Ocimum basilicum L. (OB) can efficaciously prevent endophytic contamination of Salmonella sp. and have an antibacterial effect on Salmonella sp.

Keywords: Antibacterial, endophytic, biofilm, prevention, MIC, MBC, *Ocimum*

INTRODUCTION

The *O.basilicum L.* extracts revealed an extensive and varying range of chemical compounds, depending on distinctions in chemotypes, leaf-flower colors, aroma, and origin of the plants. The chemical constituents showed the existence of monoterpene hydrocarbons, oxygenated sesquiterpene, flavonoids, oxygenated monoterpene, sesquiterpene hydrocarbons, triterpene, aromatic compounds, *etc*. The composites have been testified to demonstrate antimicrobial (Bassolé *et al.*, 2010), antifungal, antioxidant (Politeo. *et al.* 2007), antibacterial (WannissornB. *Et al.* 2005), antiviral, and insecticidal activities. The major constituents include chavicol, estragole (Hassanpouraghdam, *et al.* 2010), linalool, and eugenol (Hussain *et al.*, 2008). Abundant analysis has explored the probable risks of contamination in the vegetables during pre-cultivation and post-cultivation stages. Throughout the stages, risky inhabitants can inaugurate their habitat and get nurtured on cultivating yield. The hazard was competently increased within consequent yield whichever through promoting straight contamination or through the proliferation of surviving harmful inhabitants throughout handling and post-cultivation controlling measures (Zheng, *et al.* 2013). The principal origin of contamination in the zone is mostly noticed to be water and soil. Predictably, plants are not envisioned as hosts *Salmonella sp.*; these bacteria repeatedly concomitant among mammalian or further animal hosts. Furthermore, the latest outcomes have also revealed that *Salmonella sp.* colonize upon the exterior and innermost parts of the plants in equivalently epiphytic and endophytic survival, consistently (Kisluk and Yaron 2012). Additionally, to withstand severe environmental surroundings, it is recommended that *Salmonella sp.* could spread to the interior tissues of the plants to upgrade an effective defense versus harsh surroundings existing on the plant exterior and thus attain or improve competencies to colonize in plants and improve

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resistance to antimicrobial actions (Schikora *et al.* 2008). This provokes several sirens concerning the appliance, phenotypic behaviors, and virulence of *Salmonella sp.* concomitant with the non-animal accommodate i.e. plant. The present study was conducted to study the efficiency of preventive measures on-field application for preventing endophytic contamination of *Salmonella sp*. to the crop plants by treating the soil with eco-friendly herbal composition from *Ocimum basilicum L*. without affecting soil fertility.

MATERIALS AND METHODS

A total of 120 spinach (D) samples were collected from December 2018 to November 2019 from the regional vegetable market of the Surat city (Katargam, Varachha, Amroli, Limbayat, Adajan, Bhatar). The individual vegetable sample was kept in an individual sterile plastic zipper bag and endophytic strains of *Salmonella sp.* were acquired from the fragments of the exterior-sterilized raw salad vegetable samples. Detection and isolation were done with the help of the ISO 6579-1:2017 E method. Pathogenicity and antibiotic susceptibility assay was carried out from all the sixteen isolates (DP1 to DP16), and it was observed that five isolates from sixteen isolates showed the most pathogenic characteristics along with drug resistance and were selected for further studies. Fresh leaves of *Ocimum basilicum* L.(OB) were cleaned with tap water, shade-dried, and extracted using the soxhlet extraction method successively in methanol as a solvent. The extract suspensions were filtered through Whatman filter paper No. 1. Filtrates were then concentrated under reduced pressure at 35°C-40°C using a rotary evaporator to yield the residues of different extracts. The remaining solid was denoted as the crude extract and its percentage yield was calculated.

$$\text{Percentage Yield} = \frac{\text{Weight of crude extract obtained in gm}}{\text{Total weight of dried plant material in gm}} \times 100$$

Stocks solutions of extracts in dimethylsulphoxide (DMSO) (10.0 % v/v) were prepared and stored at 4°C for further experiments.

Antibacterial activity of OB plant extract

In vitro determination of MIC and MBC:

The antibacterial activity of the methanolic extract was investigated using the agar well diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration(MBC) of methanolic extract were determined using the Resazurin-based microtitre two-fold serial dilution method. A growth curve study was also done for confirming the antibacterial activity of OB plant extract. Bacterial growth was determined at every one-hour interval up to 10 h with the help of a UV-visible spectrophotometer. SPSS software was used to statistically analyse the data.

*Antibacterial effect study of soil treatment with MIC of OB extract in pot and field cultivation***:** Detected MIC (125 mL/L) of OB extract was prepared on large scale and two sets of the pot (20 cm X 40 cm each) for each isolate was prepared with and without OB extract, bacterial culture from isolates (DP1, DP3, DP7, DP11, DP16) were prepared according to McFarland turbidity Standard (Dastouri *et al.*, 2008) and inoculated in the soil of both the sets and observed for antibacterial effect and growth of spinach for 8 weeks. Periodically a soil, water, and spinach leave sample from each pot and spinach leaf sample was analysed for contamination of *Salmonella sp.* by ISO 6579-1:2017 E method.

Based on pot cultivation isolate DP3 was found more sensitive against MIC (125 mL/ L) of OB extract and was selected for field cultivation study. The field was selected in two plot sets (each 2 X 2 m^2 plot, 100 plants $plot^{-1}$), set one prepared with OB extract, and set two was prepared without OB extract. After treatment of soil periodically soil, water, and spinach leaf samples from fields were analysed for contamination of *Salmonella sp.* by ISO 6579-1:2017 E method. The data were statistically analyzed to determine significant treatment differences. In vitro evaluation of the antibacterial activity of OB extract against *Salmonella isolates* was conducted in the B.P.Baria science institute, Navsari, Gujarat. and field experiment was laid out in a randomized private plot at Variav, Surat in 2018-2020.

RESULTS AND DISCUSSION:

Prevalence of Salmonella sp :

Out of 120 collected spinach samples from different regions of Surat, 16 samples were found positive (Table 1) for *Salmonella sp.* and is confirmed with biochemical tests and molecular analysis. Overall 13.3% prevalence was determined and the Amroli region of Surat showed the highest % prevalence whereas Varachha, Limbayat, and Dindoli showed the lowest (1.66 %) prevalence of endophytic *Salmonella sp.* contamination. Similar results for surface inhabitants were reported by Mritunjay . *et al.* (2017). Out of all 16 *Salmonella sp.* Isolates, 4 isolates were typhoidal and the remaining 12 were nontyphoidal. Based on the results of pathogenicity tests it was observed that five isolates were most pathogenic and resistant to the most antibiotic drug. Fifteen drugs were tested for antimicrobial sensitivity out of which resistance for nine drugs was exhibited by isolate DP3. Whereas DP1 isolates showed drug resistance for seven drugs. Based on pathogenicity assays it was found that out of all isolates DP3 was most pathogenic as they showed strong biofilm production, hemolysin production, salt aggregation, crystal violate binding, autoagglutination, swarming, EPS production, congo-red assay, and most drug-resistant, so DP3 isolates were selected for lab-scale, pot, and field cultivation experiments. OB extracts were prepared in methanol solvents. The percentage yield of these crude extract was 36.2% w/w.

Table: 1 Location-wise % prevalence of *Salmonella sp.*

Location	Total spinach sample collected	Number of *Salmonella sp.* isolates	% Prevalence* of *Salmonella sp.*	Isolates labeled as
Katargam	20	3	2.5	DP1-DP3
Varachha	20	2	1.66	DP4-DP5
Amroli	20	4	3.33	DP6-DP9
Adajan	20	3	2.5	DP10-DP12
Limbayat	20	2	1.66	DP13-DP14
Dindoli	20	2	1.66	DP15-DP16
TOTAL	N = 120	16	13.33	DP1-DP16

**% prevalence = (total number of isolates /total number of samples analysed) X 100*

Antibacterial activity of OB plant extract
*In vitro analysis***:**

The zone of inhibition (ZI) of various OB extract aliquots and ampicillin against 5 bacterial isolates were recorded at different concentrations. The various calculated ZI of all the OB extracts dilution ranges from 12.2 ± 0.1 mm to 26.1 ± 0.4 mm (Table 2).

Table: 2 Agar well diffusion assay zone of inhibition

Dilution µl /mL	OB extract ZI (mm ± SD)					Ampicillin ZI (mm ± SD) STANDARD
	DP1	DP3	DP7	DP11	DP16	
20	14.2 ± 0.1	18.2 ± 0.1	12.2 ± 0.1	14.6 ± 0.1	16.2 ± 0.1	16.1± 0.1
40	14.6 ± 0.4	18.6 ± 0.4	12.6 ± 0.4	18.2 ± 0.4	16.6 ± 0.4	18.1 ± 0.1
80	16.1 ± 0.2	18.8 ± 0.2	14.8 ± 0.2	18.7 ± 0.2	16.8 ± 0.2	20.4 ± 0.2
100	16.3 ± 0.4	20.2 ± 0.4	16.2 ± 0.4	18.8 ± 0.4	18.2 ± 0.4	22.4 ± 0.4
500	16.6 ± 0.4	26.1 ± 0.4	20.1 ± 0.4	22.1 ± 0.4	21.1 ± 0.4	24.6 ± 0.6

**ZI= zone of inhibition*

Similar results were reported by Rattanachaikunsopon P. et. al. (2010). DP3 isolates showed the highest zone size 26.1 ± 0.4 mm for aliquotes 500 µl/ml whereas DP7 isolates showed lowest zone size 16.6 ± 0.4 mm for aliquotes 500 µl/ml. DP3 isolates showed significant zone size 18.2 ± 0.1 mm observed for lowest dilution was 20 µl/ml. Al-Hadidy Y. I. et. al.(2019) had reported similar results for *Salmonella sp.* against *Eucalyptus*

camaldulensis essential oil. Out of all five isolates, DP3 showed strong sensitivity against OB extract at all dilutions whereas other isolates showed mild to moderate sensitivity similar results were reported by Mostafa, A.A *et.al.* (2018). According to Resazurin-based micro serial dilution crude OB extract, the highest antibacterial activity against DP3 isolates with MIC value of 125 µl/ml and MBC value 250 µl/ml was observed(Table3). Whereas lowest activity was observed against DP16 isolate with MIC value of 500 µl/ml. Based on the comparison of MIC values of OB extract dilutions DP3 isolate was selected for pot and field cultivation experiments. These results indicated that OB extract can inhibit bacterial growth without bactericidal activity as the MIC value is lower than the MBC value.

Table : 3 Resazurin based micro-titre MIC assay

ISOLATES	500 µl /mL	250 µl /mL	125 µl /mL	62.5 µl /mL	31.25 µl /mL	15.62 µl /mL	7.81 µl /mL	3.90 µl /mL
DP1	NG	NG	G	G	G	G	G	G
DP3	NG	NG	NG	G	G	G	G	G
DP7	NG	G	G	G	G	G	G	G
DP11	NG	NG	G	G	G	G	G	G
DP16	NG	G	G	G	G	G	G	G

**G=Growth (pink colour), **NG = No growth(blue colour)*

Growth curve analysis with MIC:

This study showed that treatment with MIC of OB Extract can successfully inhibit the growth of different *Salmonella sp*. isolates. Out of all five isolates, DP3 showed more sensitivity against OB extract when treated with MIC (125 µl /mL) (Figure: 1) and is selected for further studies. Similar results were reported for *Citrus hystrix* against *Salmonella sp*. by Maulidah, E. et. al (2021). Growth curve pattern showed DP3 growth was inhibited without bactericidal activity with MIC concentration of OB extract and can not form biofilm components in presence of OB extract when assayed for crystal violate binding assay, whereas other isolates prevented from growth but they did not found to be prevented from biofilm production when treated with MIC of OB extracts. These results indicate there is a significant growth inhibitory effect of OB extract on all the isolates. Out of all isolates, DP3 showed the highest inhibitory effect against OB extract.

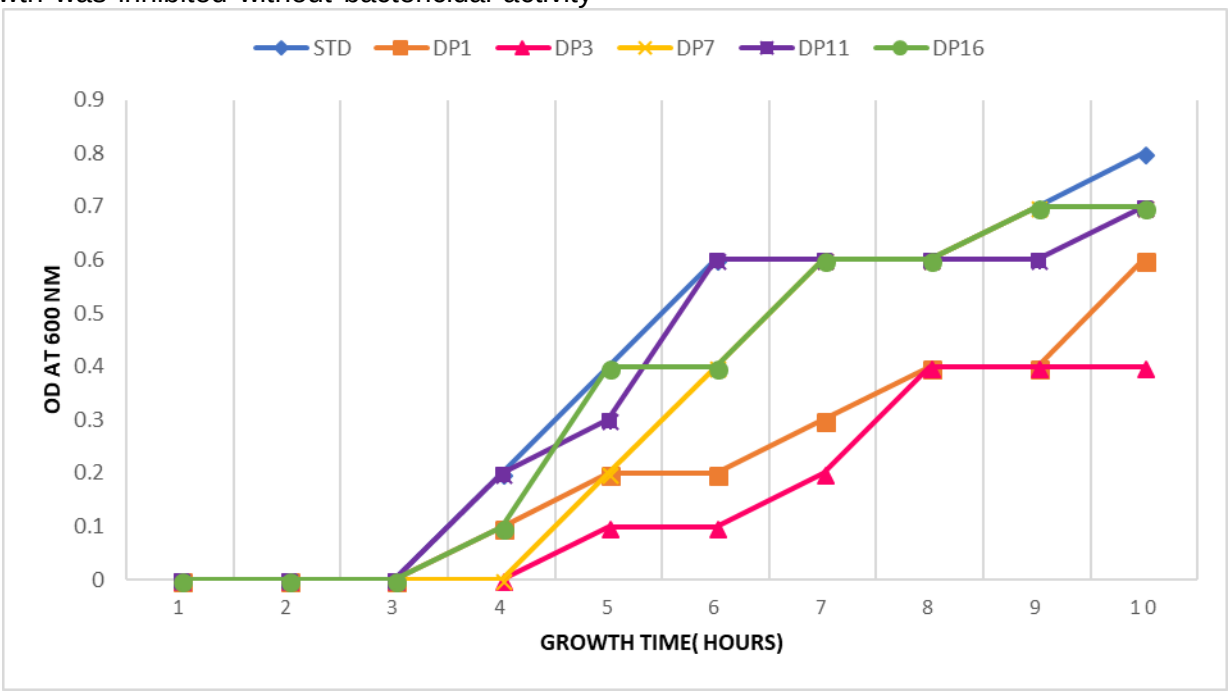

Figure 1: Growth curve study of *Salmonella sp. with* MIC of OB extract

Antibacterial effect study with MIC of OB extract in pot and field cultivation

On the treatment of soil with MIC concentration of OB extract to set - 1, it was observed that *Salmonella sp*. can not contaminate spinach plant in presence of OB extract. Periodically, on each 14th day three random samples of soil, water, and spinach leaf were collected from both sets and were analysed for contamination of *Salmonella sp.* for subsequent 56 days. From set- 2 viable *Salmonella sp*. was recovered from soil and spinach leaves both, whereas from set- 1 viable *Salmonella sp. was* recovered only from soil, which was treated with MIC of OB extract (Table 4).

Table: 4 Antibacterial effect study of MIC of OB extract on pot and field cultivation

Periodic bacterial detection	Pot experiment				Field experiment			
	SET -1		SET- 2		PLOT-1		PLOT -2	
	Soil treated with MIC of OB Extract	Harvested spinach leaves	Soil without treatment	Harvested spinach leaves	Soil treated with MIC of OB Extract	Harvested spinach leaves	Soil without treatment	Harvested spinach leaves
0 day	SD	-	SD	-	SD	-	SD	-
14 day	SD	SND	SD	SD	SD	SND	SD	SD
28 day	SD	SND	SD	SD	SD	SND	SD	SD
42 days	SND	SND	SD	SD	SD	SND	SD	SD
56 days	SND	SND	SD	SD	SND	SND	SD	SD

****Above experiment conducted thrice, *SD = Salmonella sp. detected **SND = Salmonella sp. not detected*

These results indicate that *Salmonella sp*. in set -1 can not contaminate the spinach plant in presence of OB extracts. On the treatment of soil with OB extracts host- parasitic interaction inhibited and along with this as their growth is inhibited they were not able to form biofilms so *Salmonella sp.* can not contaminate spinach plant though they were found viable in the soil. It is also observed even after 56 days of the soil treatment effect of OB extract is effective against *Salmonella sp.* On the treatment with MIC concentration of OB extract to the soil of plot-1, it was observed that *Salmonella sp*. can not contaminate spinach plant in presence of OB extract even after 56 days of exposure. Periodically soil, water, and spinach leaf sample were taken and analysed for contamination of *Salmonella sp.* from plot-1 and plot- 2. from plot-2 viable *Salmonella sp*. was recovered from the soil on each periodic detection and spinach leaves both, whereas from the plot- 1 viable *Salmonella sp.* was recovered only from soil, which is treated with MIC of OB extract (Table 4). These results indicate that *Salmonella sp*. in plot- 1 can not contaminate the spinach plant in presence of OB extracts even after 56 days of exposure.

From the above experiments, it can be concluded that MIC (125 µl /mL) of OB extract can successfully treat the soil for preventing contamination of *Salmonella sp*. to spinach plants. OB plant can be used as a preventive measure to overcome soil contamination from *Salmonella sp.* which may lead to improvements in the health of salad vegetable crops.

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