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Endophytic Bacteria from *Ocimum sanctum* and Their Yield Enhancing Capabilities

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Abstract Endophytes are beneficial microbes that reside intercellularly inside the plants. Interaction of endophytes with the host plants and their function within their host are important to address ecological relevance of endophyte. Four endophytic bacteria OS-9, OS-10, OS-11, and OS-12 were isolated from healthy leaves of *Ocimum sanctum.* These isolated microbes were screened in dual culture against various phytopathogenic fungi viz. *Rhizoctonia solani, Sclerotium rolfsii, Fusarium solani, Alternaria solani*, and *Colletotrichum lindemuthianum.* Of these, strain OS-9 was found to be antagonistic to *R. solani, A. solani, F. solani*, and *C. lindemuthianum* while OS-11 was found antagonistic against *A. solani only.* The growth-promoting benefits of the endophytes were initially evaluated in the glasshouse by inoculated seeds of *O. sanctum*. Treatment with endophytes OS-10 and OS-11 resulted in significant enhancement of growth as revealed by increase in fresh as well as dry weight. Further, field trials involving two genotypes OS Purple and CIM-Angana were conducted with strains OS-10 and OS-11. The growth-promoting effect was visible on both the genotypes tested as the endophytes significantly enhanced fresh herbage yield (t/ha). Interestingly, these endophytes increased the content of essential oil particularly in cultivar OS Purple and thereby increasing the total oil yields. Molecular characterization of strain OS-11 indicated the strain to be highly related to the type strain of *Bacillus subtilis.*

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Introduction

Tulsi (*Ocimum sanctum*) belonging to family *Lamiaceae* is a widely grown and sacred plant of India bearing high medicinal value. The primary places of origin of *O. sanctum* is believed to be Central and West Africa [20]. Most of the species of *Ocimum* grow throughout the tropical and subtropical regions of the world [2]. Tulsi leaves contain a bright yellow volatile oil, reported to possess anti-bacterial properties and also acts as insecticide. Tulsi leaves are traditionally used for getting relief from common cold, bronchitis, cough, and digestive problems.

The number of reports on bacteria being isolated from inside healthy plant tissues considered as endophytes is fastly increasing [1, 4, 6, 10, 11, 13, 27]. The internal tissues of the plants provide a uniform and safe environment for the endophytes. This advantage envisages the use of endophytic bacteria for more successful biological control of plant diseases [7, 8, 17, 18, 24, 26]. Although the interaction between endophytic bacteria and their host plants is not fully understood, many strains have been shown to promote plant growth [5, 6, 11]. Some of endophytes may be producing bioactive substances that may have applicability in medicine. Therefore, the study of host colonizing microbes stimulating plant growth either directly by producing plant hormones, nutrient uptake and resistance to the stress conditions or indirectly by changing the microbial balance in the rhizosphere in favor of these beneficial microbial organisms could open a fascinating area of research in medicinal plants where only fragmented information in terms of endophyte occurs. The present study focuses on the isolation and characterization of some of the endophytic bacteria from *O. sanctum* for their yield enhancing capabilities both in terms of herbage and essential oil yields.

Materials and Methods

Endophytic bacteria were isolated from the healthy and asymptomatic leaves of *O. sanctum* var. CIM-Angana. The leaves were kept under running tap water for washing for 10 min and were then placed in 1% sodium hypochlorite (NaOCl) for 10 min and rinsed 4 times in 0.02 M sterile potassium phosphate buffer (PB) pH 7.0. A 100 µl of aliquot was taken from the final buffer wash and transferred to 5 ml nutrient broth in the screw cap bottle with the control as sterility check. Samples were discarded if the growth was detected in the sterility check samples in nutrient broth (NB) kept in an incubator shaker (200 rpm at 28°C) after 48 h. Each sample was then macerated in a sterile pestle and mortar with sterile distilled water. 100 µl of the extract was taken and serial dilutions upto 10^{-5} of the titurate were made. Each dilution of the sample was plated on three different media: nutrient agar (g/l; peptone-5, Beef extract-2, agar-20, pH 5.0), King's B medium (g/l protease peptone-20, K_2HPO_4-1.5, $MgSO_4 \cdot 7H_2O$-1.5, glycerol-20 ml, and agar-15, pH 7.0) and potato dextrose agar (g/l; potato infusion-200, dextrose-20, agar-15, pH 5.6)with three replications each. The plates were incubated at 28°C for 48–72 h. A population of 6.3×10^5 to 3.7×10^6 was detected on nutrient agar plates while no colonies appeared on King's B and potato dextrose agar media. A representative of each bacterium, as evident from their colony morphology was transferred to fresh nutrient agar medium plates to establish pure cultures of endophytic bacteria. A total of four bacteria apparently looking different by their cultural characteristics were isolated.

In Vitro Antagonism of Endophytes Against Plant Pathogenic Fungi

A 9 mm PDA culture disc from the plates of *Fusarium solani, Rhizoctonia solani, Sclerotium rolfsii*, *Colletotrichum lindemuthianum, Alternaria solani,* growing in petri dishes was cut individually from 7-day-old culture. This was placed on one side of the previously plated sterilized modified PDA medium (g/500 ml PDA (Hi Media)-19.5, peptone-1, yeast extract-0.5, agar-2.5) approximately 1.5 cm away from the edge of the plate. Simultaneously, the endophytic bacteria were streaked onto the opposite side of the petri plate. Three replications of each treatment and suitable controls were maintained. The plates were incubated at 28°C for 7 days.

Glasshouse Study

Nursery of *O. sanctum* was raised in sterilized soil in earthen pans (12″ diameter). Seeds of *Ocimum* were surface sterilized with 10% NaOCl for 1 min, rinsed twice with distilled water. Culture of individual isolate of all the four bacterial endophytes, were grown in nutrient broth for 48 h on an orbital incubator shaker (200 rpm at 28° C). Bacterial cells were harvested by centrifuging at 10000 rpm for 10 min in a refrigerated centrifuge. The supernatant was discarded and pellets were diluted distilled water. The surface sterilized seeds then dipped into the prepared inoculums (cfu $1.3–2.1 \times 10^{8)}$ of endophytes for 1 h. The seeds then transferred to the pans along with respective culture. The 45-day-old seedlings were transferred to 9″ diameter earthen pots containing sterilized soil: FYM (2:1). The seedlings before transplanting were tested (at least 5 from each pans) for presence of inoculated endophytes. The plants were maintained and harvested after 60 days at the time of initiation of flowering. Three replications were maintained.

Field Study

Field trials were conducted during 2007–2008 with two best performing endophytes viz. OS-10 and OS-11 as observed during glasshouse studies. Two varieties of *O. sanctum* viz. OS Purple and CIM-Angana were included with three replications. The 45-day-old seedlings raised in sterile soil along with endophytes OS-10 and OS-11 (as described earlier) were transplanted in field. Each treatment plot (9 m^2) consisted of six rows of six plants each. Data on fresh herbage yield were recorded after 3 months at harvesting. The herbage was distilled for essential oil in Clevenger apparatus to determine the content of essential oil in plants. Data were subjected to analysis of variance (ANOVA), the least significant difference test was applied to make comparisons among the means ($P < 0.05$).

Molecular Characterization of Selected Endophyte (OS-11) from *O. sanctum*

The strain OS-11 was sent to Institute of Microbial Technology, Chandigarh, India for identification based on 16S rRNA gene sequence. The phylogenetic position was inferred using the Neighbor-Joining method [23]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.

Results

The isolated endophytic bacteria were also tested for antagonism. Of these, strain OS-9 was found to be

antagonistic to *R. solani, A. solani, C. lindemuthianum*, and *F. solani* while OS-11 was found to be antagonistic to *A. solani* only. The other endophytic bacteria were not antagonistic to any of the fungus tested.

Potential of Isolated Endophytes for Their Growth Enhancing Capabilities in *Ocimum* (Glasshouse Study)

The results of the glasshouse experiment conducted to assess the potential of the endophytic bacteria for their yield enhancement showed that strains OS-10 and OS-11 significantly improved the fresh weight as well as dry weight over control (Table 1), an increase of about 27 and 24%, respectively (Fig. 1). The other two endophytes could not increase the total herb and therefore only OS-10 and OS-11 were included for field trials. The endophytes could be reisolated from the inoculated seedlings; their population ranging from 5.7×10^5 to 3.1×10^6/g leaf tissue after 45 days of inoculation.

Table 1 Determining the potential of isolated endophytes for their growth enhancing capabilities in *Ocimum sanctum* (glasshouse experiment)

Endophyte	Fresh weight (g/plant)	Dry weight (g/plant)
OS-9	15.1	3.3
OS-10	21.7	4.8
OS-11	21.2	4.5
OS-12	17.1	3.4
Control	17.1	3.8
LSD ($P = 0.05$)	2.91	0.61

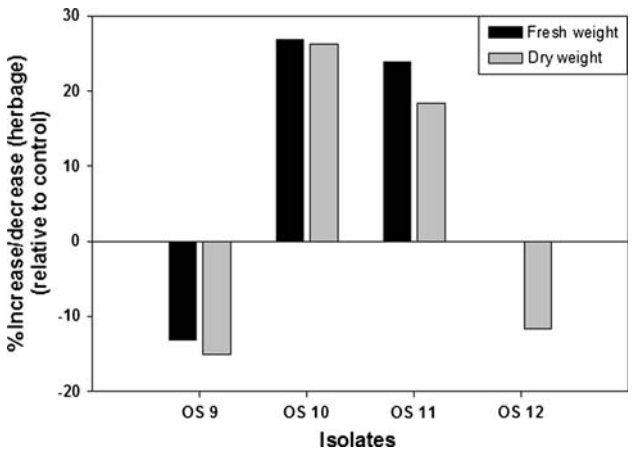

Fig. 1 Herb yields of endophyte inoculated *Ocimum* plants relative to uninoculated control (glasshouse experiment)

Potential of Isolated Endophytes for Their Yield Enhancing Capabilities in *O. sanctum* (Field Study)

Field experiment conducted to evaluate the performance of OS-10 and OS-11 on growth and yield of *O. sanctum* clearly indicated that the endophytes OS-10 and OS-11 have potential to increase the total herb yields. This effect was visible on both the cultivars OS Purple and CIM-Angana. Interestingly, the endophyte OS-11 also increased the content of essential oil in both the cultivars more markedly in OS Purple and thereby increasing the total oil yields (Table 2). The effects being more pronounced in the cultivar OS Purple with the endophyte OS-11 (Fig. 2). On the other hand endophyte OS-10 was more effective in improving yields in OS Purple. No significant differences were observed in the severity of leaf blight, which although did not appear in severe form between endophyte inoculated and uninoculated treatments.

Molecular Characterization of Selected Endophytes (OS-11) from *O. sanctum*

The endophyte was subjected to molecular characterization for the purpose of identification based on 16S rRNA gene sequence. It was observed that strain OS-11 showed 100% similarity with the sequence of 16S rRNA gene of *Bacillus subtilis* (AJ 276351). The sequence of OS-11 has been deposited to NCBI databank (QG461751). The culture has been deposited with Microbial Type Culture Collection, Chandigarh, India (MTCC 10010) and is available to public.

Discussion

This study determines the useful effects of endophytes in *O. sanctum*, an important medicinal plant. Endophytes were isolated from the leaves of the naturally soil grown *Ocimum* plants and the attempts to reisolate endophytic bacteria from surface sterilized seeds were unsuccessful thus indicating the need of reinoculating it with isolated endophytic cultures. The present study has shown that an endophyte OS-11 isolated from *O. sanctum* and identified as *Bacillus subtilis* showed significant enhancement in the herb yields. Endophytes may promote plant growth by a number of mechanisms such as phosphate solubilization activity [14, 26, 27], nitrogen fixation [16, 22], and the production of siderophore [9]. Endophytes have also been shown to perform antagonistic activity against phytopathogen [3, 5, 6, 11, 19, 21] and are known control plant disease by induction of systemic resistance and other defense responses including the production of phytoalexins, accumulation of pathogenesis related proteins,

Table 2 In vivo determining the potential of isolated endophytes for their yield enhancing capacity in *Ocimum sanctum*

Endophyte	Fresh herbage yield (t/ha)		Oil content (%)		Oil yield(kg/ha)	
	OS Purple	CIM-Angana	OS Purple	CIM-Angana	OS Purple	CIM-Angana
OS-10	10.28	9.67	0.03	0.04	3.45	3.52
OS-11	9.26	9.16	0.04	0.03	4.96	3.09
Control	7.22	6.56	0.02	0.03	1.67	1.77
LSD ($P = 0.05$)	1.03	1.12	0.01	0.01	1.13	1.21

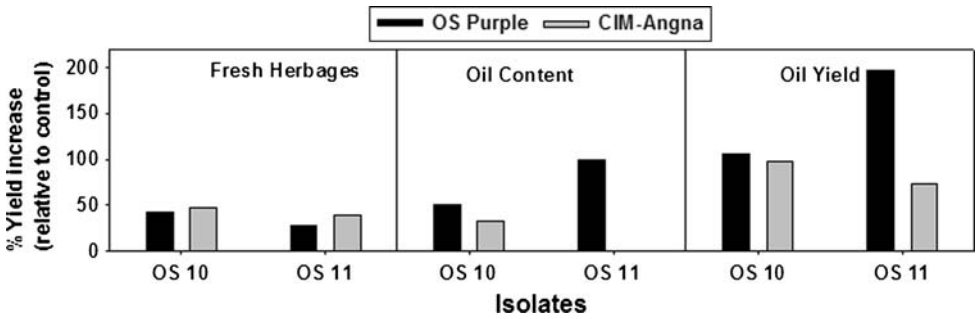

Fig. 2 Crop yields of endophyte inoculated *Ocimum* plants relative to uninoculated control (field experiment)

deposition of structural barriers in the cell wall of the host plant, and by the production of antimicrobial compounds and siderophore [15]. However, endophytic microorganisms were not selected on the basis of their antagonistic potential but because of their growth-promoting potential as no significant damage to *Ocimum* are generally observed at this place. During the present field studies also, leaf blight generally affecting *Ocimum* in India [25] did not appear in severe form, during the period of study (2007–2008). The present study has also demonstrated that the endophytic bacteria may play a major role in secondary metabolite production as these endophytes especially OS-11 in OS Purple could significantly enhance the content of essential oil and hence oil yields. Although no serious diseases were observed both in endophyte inoculated and uninoculated plants, these endophytes may provide substantial protection against plant pathogen in areas with records of epidemics. This may be particularly important in case of medicinal plants where application of pesticides is not desirable. The characterization of endophytes through molecular means indicated its close homology to *B. subtilis*. *Bacillus subtilis* and *B. licheniformis* have been reported as plant endophytes [12, 28].

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