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IN VITRO SELECTION OF MICROBIAL PHYTOHORMONE ON PLANT REGENERATION OF *OCIMUM SANCTUM* L. AND ITS SECONDARY METABOLITES PRODUCTION

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ABSTRACT
In vitro micropropagation is an effective tool for rapid multiplication of species in which it is necessary to obtain a high progeny uniformity. *Ocimum sanctum*, commonly known as "Holy Basil", belongs to the family of Lamiaceae. The main aim of this study was to investigate the possible role of indole acetic acid (IAA) from bacteria to induce *in vitro* adventitious shoots in inter nodal explants of *Ocimum sanctum* L. In this research, two successive experiments were performed: first, the effects of explants source on MS medium supplemented with four different plant growth hormones of IAA, NAA, BAP and KIN in different concentrations were studied either individually or in combination and second, the effects of different levels of microbial IAA growth hormone with BAP. An account of this, the callus was initiate in MS medium supplemented with 0.2 mg/L NAA + 0.5 mg/L KIN and 0.4 mg/L IAA + 0.5 mg/L BAP for two weeks. Similarly, Microbial 0.2 ml IAA +0.5ml BAP mg/L increasing the shoot proliferation for one week at the average length of shoot (2.29 ± 0.32 cm). The *in vitro* callus and microbial *in vitro* eluted callus are used to compare their compound by HPLC and GC MS analysis. This result paved the way for *in vitro* and *in vivo* analysis in the further step of drug designing.

KEYWORDS: *Ocimum sanctum*, Plant growth hormones, Proliferation, HPLC and GC MS.

INTRODUCTION
Medicinal plants are valuable sources of traditional medicine. Genus *Ocimum* contains more than 150 species, collectively called as 'Basil'. *In vitro* micropropagation is an effective mean for rapid multiplication of species in which it is necessary to obtain a high progeny uniformity.[1] As discovered by various researches, even close members of the same genus (*Ocimum*) do not possess the same chemical constituents revealed that as *O. sanctum* is able to cross pollinate with other plants of the similar genus, certain plants would not be true-to-type, and if there are genetic variations in the plant, the chemical constituents would be different.[2] This is where plant tissue culture could be applied and helps to solve the problem, as plant tissue culture produces offspring that are identical to the parent plant.

MATERIALS AND METHODS
Sample Collection
Young leaf, stem of *Ocimum sanctum* (Tulsi) was selected from nearby places of the garden in Sivakasi, Virudhunagar District and collected from with sterile blades.

Phytochemical Screening
The phytochemical screening of fresh aqueous extracts of *Ocimum sanctum* was performed for the presence of various phytoconstituents.[3]

Antibacterial Activity
The aqueous *O. sanctum* extract was screened for biological activity against the test organisms *Bacillus* cereus, *Pseudomonas*, *E. coli*, *Salmonella*, and *Staphylococcus*. The 24 hours old cultures of test organisms were inoculated by making a lawn on Muller Hinton Agar by using sterile cotton swab. Well was cutted by standard well puncture. The different concentration of aqueous extract was added to the well such as 25µl, 50µl, 75µl and control (distilled water). The plates were incubated 37˚C for 48 hours. The inhibition zone was measured. All processes were performed aseptically, to avoid contamination.

Microbial IAA Production
The IAA producing bacteria *Bacillus* cereus KR9 [4] was used to produce Indole 3 acetic acid. The organisms were grown in yeast malt dextrose broth (YMD broth) with L-tryptophan and incubated at 28°C for 4 days.

Quantification of Microbial IAA

After incubation the broth was centrifuged. Supernatant was reserved and 1ml was mixed with 2ml of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HCLO_4$ solution) and kept in the dark. The optical density (OD) was recorded at 530 nm after 30 min against the standard IAA solution.

Extraction and purification of IAA

Isolates were cultivated in YMD broth and it was centrifuged at 5000 rpm in 15 min. The supernatant was collected and mixed with ethyl acetate (1: 2). After vigorous shaking it was allowed to stand for 10 min. IAA was extracted within solvent layer. The procedure was repeated 3 to 4 times.

Micropropagation

Surface Sterilization

The explants were sterilized by standard protocol such as the explants were placed under running tap water for about one hour to remove foreign contaminations. During washing surfactant Tween-80 along with detergent was also added to make the contact of water deep in the plant material. Subsequently the explants were taken to laminar airflow cabinet for further sterilization. After rinsing explants were dipped in 70% ethanol for 30 seconds and then were treated with 0.1% mercuric chloride ($HgCl_2$) and 5% sodium hypochloride for 5-6 minutes simultaneously. After this treatment, explants were given 4-5 thorough washing with autoclaved distilled water to remove any trace of the surface sterilants, under aseptic conditions.

Media Preparation (MS Media)

The MS media (42.8 gm/lit) and $CaCl_2$ (440 mg/lit) was dissolved in 1000 ml of sterile double distilled water and subjected to adjust the pH 5.8. Thereafter, there was addition of agar powder (8 gm/lit) dissolved in the prepared media solution. However, after dissolution of agar, 20 ml media was poured into sterile tissue culture tubes and bottles. The tubes and bottles containing media were subjected to autoclaving at 15 lb pressure, at 121˚C temperature for 15 min. After autoclaving the test tubes were allowed to solidify for about 60 minutes, contamination and then inoculated by proper explants. Observations were recorded time to time and all the culture tubes and bottles were kept under observation.

Effect of Plant Growth Hormones on Cultured Explants

Explants such as leaves and nodal shoot were surface sterilized and inoculated in MS Media with various concentrations of IAA, NAA, BAP and KIN alone and in combinations with various concentrations of IAA, NAA, BAP and KIN and were kept in controlled aseptic conditions.

Inoculum Conditions

All the cultures were maintained in the culture room at 25±1ºC, under photoperiod provided by white fluorescent tubes with the intensity of 3000lux. The cultures were incubated for 30 days and daily observations were made to monitor the day of initial callus formation.

Adventitious Shoot by Microbial IAA Phyto hormone

Inter nodal explants inoculated with culture supernatant of *Bacillus cereus* KR9 in different concentration such as 0.2 -1.0ml combines with 0.5 ml of BAP.

SDS – PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis is a technique used in biochemistry, genetics and molecular biology to separate proteins according to their electrophoretic mobility. The SDS-PAGE was carried out using method of using 12% (w/v) separating gel and and 4% (w/v) stacking gel.[21] The protein concentrations of the *In vitro* and *in vivo* cultured were measured according to the method of.[20] In the first well molecular marker was included. In the third well 20 µl of *in vivo Ocimum sanctum* leave sample was included. In the next well 20 µl of *in vitro* cultures leave sample was added and the last well 20 µl of microbial *in vitro O. sanctum* leave sample was added.

Estimation of Secondary Metabolites By Hplc

HPLC analysis

A Shimadzu LC 10 AT VC system consisting of a Binary pump connected to a fractional collector (FRC – 10 A) and system controller (SCL – 10 A VP) was used to perform HPLC analysis. A reverse phase Varian C18 column (5Rm particle size, 25 cm x 0.25 Rm) was used. The detection was carried out at 280 nm using a photodiode array variable detector (SPD – M 10 A VP). Mobile phase solution A consisted of 0.1% TFA (trifluoroacetic acid) in water and absolute acetonitrile was used as solution B (HPLC grade). For the separation of individual compounds, the mobile phase utilizing the isocratic (30: 70) solvent elution over a total run time of 25 minutes, with an injection volume of 15 Rl and a flow rate of 1 ml / min and pressure maximum of 220 Ibs. Unknown constituent present in each sample were identified by using retention time matching.

Gas Chromotography Mass Spectrometery Analysis

GC-MS analysis was carried out on a Perkin Elmer Clarus - 500 gas chromatograph fitted with a fused silica capillary column (25 m x 0.25 mm film thickness 0.33 mm) crosslinked with 5 % phenyl methyl silicone with a split / Spitless injector was used to separate the *Ocimum* sanctum extracts. The oven temperature was programmed from 160 0 - 220˚at 5˚C / min. Helium was used as carrier gas at a flow rate of 2 ml / min. Inlet and detector temperature were 200˚C and 240˚C respectively. Sample volume of 2 µl was injected in the split mode (30:1) into the gas chromatograph. Turbo mass Software is used to interlink the instrument and computer. The identification of volatile compounds from the samples was based on retention indices (RI) and computer matching with Spectral library available with instrument

(NIST). The MS operating parameters were as follows ionizations voltage 70 eV and ion source temperature 200˚C. Retention indices (RI) values were measured on Elit -5MS column. For RI calculation, a mixture of homologues n- alkanes was used under the same chromatographic condition, which was used for the analysis of the plant volatiles.

RESULTS

Phytochemical screening

The result of the preliminary phytochemical screening was carried out on the aqueous extracts of the *Ocimum sanctum L.* sample and revealed the presence of a wide range of phytoconstituents including alkaloids, glycosides, saponins, flavonoids, tannins, steroids.

Antibacterial activity

The well diffusion methods showed the better results as clear zone was observed around the wells with high concentration of aqueous extract of *Ocimum sanctum L.* against all test pathogens as showed in table 1.

Quantification of Microbial IAA Production

Bacillus cereus KR9[4] produced IAA in YMD broth with L- tryptophan. Hence optical density was measured to be 1.319 at 530nm compared to standard graph, the *Bacillus cereus* KR9 produce IAA in the range of 0.9ml concentration of standard IAA.

Analysis of IAA by TLC

Purified IAA sample was compared with standard IAA on TLC chromatograms. TLC of ethyl acetate extract showed pink colour spot at the Rf corresponding to the authentic IAA (0.58) and test sample (0.53). This result showed the *Bacillus* cereus KR9 had the potential to producing IAA.

Effect of growth regulators on Micropropagation

The best shoot initiation and proliferation was done in different concentration of phytohormones as shown in the table 2 and Fig 1.

Adventitious Shoot by Microbial IAA Phytohormone

The internodal explants were initiate in the MS media with Bacterial Supernatant with different concentrations. In these different microbial concentrations, 0.2 ml IAA mg/L + 0.5 ml BAP mg/L showed the best average shoot length 2.29±0.32cm within a week.

Analysis of protein profiles by SDS – PAGE

Protein contents were identified using SDS – PAGE with different *in vitro* and *in vivo* samples. *In vivo Ocimum* leaf sample showed the high molecular weight band 120 kDa. *In vitro* culture leaves of *O. sanctum* showed the molecular weight of 114 kDa and the Microbial *In vitro* leaves showed 90kDa molecular weight Fig 2.

Secondary metabolites production by HPLC

Secondary metabolite compounds were eluted from the different plant samples of *O. sanctum in vitro* callus cultures. 0.4 ml of IAA + 0.5 ml of BAP mg/L cultured *in vitro* callus (Fig. 3) and the microbial eluted 0.2 ml of IAA + 0.5 ml of BAP mg/L of *in vitro* callus (Fig. 4) were used to identify the metabolites.

Gas Chromotography Mass Spectrometery Analysis

GC-MS analysis was done for the ethanol extract of the combinations of microbial 0.2 ml of IAA + 0.5ml of BAP mg/L of *Ocimum sanctum in vitro* callus cultures. It showed eight major peaks (Fig 5) and has been identified after comparison of the mass spectra with NIST library, indicating the presence of nineteen phytocomponents. From the results, the eluted compounds were observed.

Table 1: Antibacterial activity of extract against test organisms.

S.No.	Test Organisms	Zone of Inhibition(mm)			
		25 µl	50 µl	75 µl	Control
1	*Bacillus cereus*	11mm	15mm	22mm	-
2	*Pseudomonas*	10mm	15mm	18mm	-
3	*E.coli*	-	14mm	21mm	-
4	*Salmonella*	-	13mm	17mm	-
5	*Staphylococus*	-	09mm	15mm	-

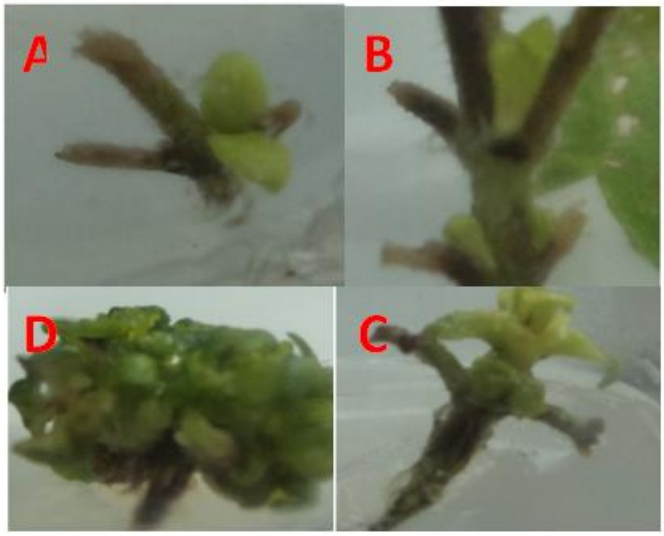

Fig. 1: Multiple shoot proliferation.

A - 0.5ml of IAA, B - 3 ml of NAA
C - 0.4ml of IAA mg/L + 0.5ml of BAP
D - 0.2 ml of microbial IAA mg/L + 0.5 ml BAP mg/L

Table 2: Effect of growth regulators on Micropropagation.

S.No	Phytohormones concentration (mg/L)	Average shoot length (cm) ± SD
1	0.5ml of IAA	0.36 ± 0.61
2	3 ml of NAA	0.16 ± 0.34
3	0.5ml of BAP	0.10 ± 0.54
4	0.5ml of KIN	0.09 ± 0.34
5	0.4ml of IAA mg/L + 0.5ml of BAP	0.81±0.46
6	0.2ml of NAA mg/L + 0.5ml of KIN	1.39 ± 0.85

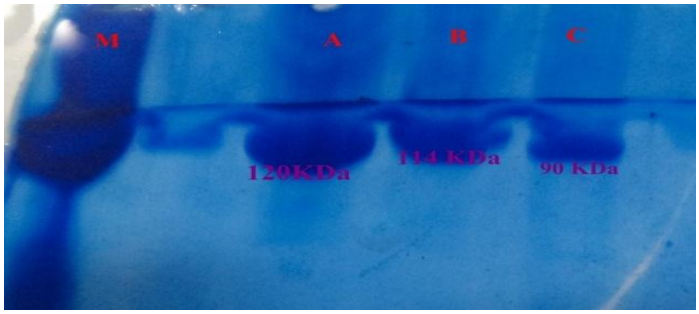

Fig. 2: Protein profile analysis by SDS PAGE.

M – Marker protein, A - *in vivo Ocimum sanctum* leaves
B - *In vitro* cultures leaves of *O. sanctum*, C - Microbial *in vitro O. sanctum* leaves

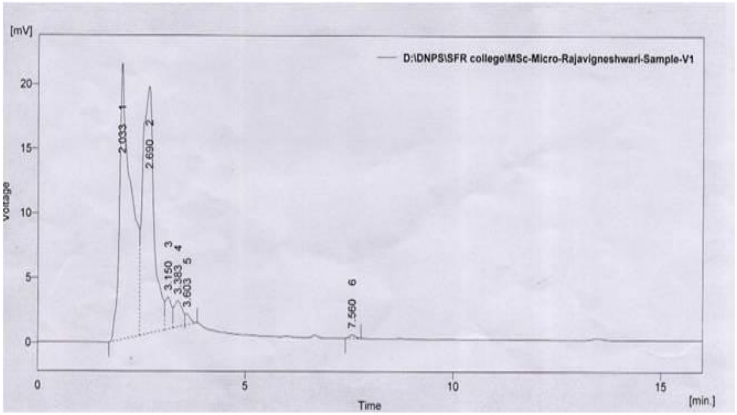

Fig. 3: Secondary metabolites for *in vitro* IAA (0.4 ml of IAA + 0.5 ml of BAP mg/L) initiated callus.

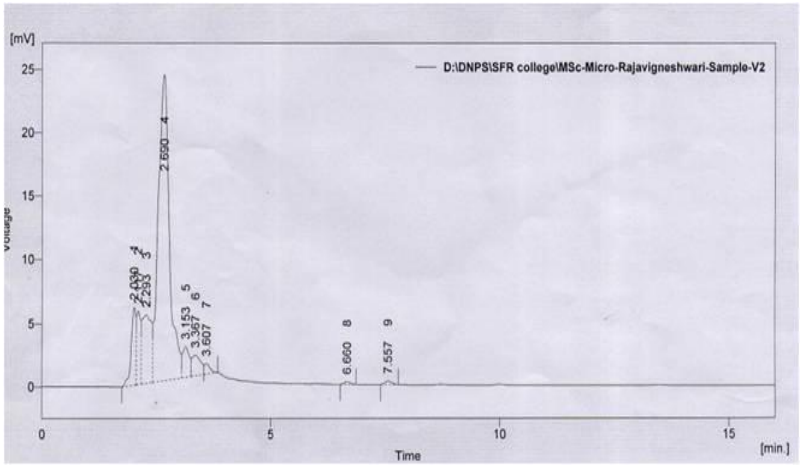

Fig. 4: Secondary metabolites for Microbial *in vitro* eluted IAA(0.2 ml of IAA + 0.5 ml of BAPmg/L) initiated callus.

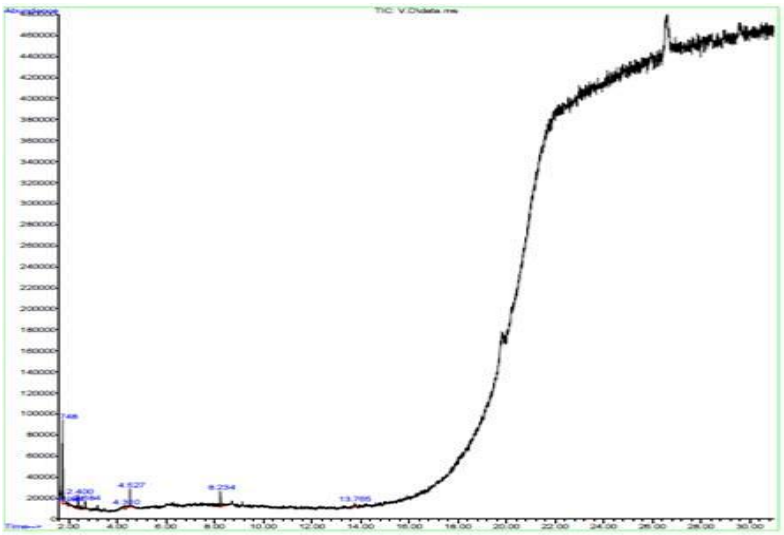

Fig. 5: GC MS analysis of Microbal *in vitro* eluted callus.

DISSCUSION

In the present study *Ocimum sanctum* plant showed the presence of different phytoconstituents like alkaloids, glycosides, saponins, flavonoids, tannins, steroids. In earlier[9] reported the compounds alkaloids, steroids, phenolic compounds, tannins, saponins, carbohydrates, proteins, amino acids and organic acids. Likewise[12] stated the presence of phenolic compounds, tannins, flavonoids, cardiac glycosides, and alkaloids were present in *Ocimum sanctum*. In this study aqueous extract of *Ocimum sanctum* L. showed the best antibacterial activity against the pathogens like *Pseudomonas*, *Salmonella* and *Staphylococus*. Clear zone was observed around the wells with high concentration of 75 µl like 18 mm, 17 mm and 15 mm. Likewise[13] reported that the Methanolic extract of *Ocimum sanctum* showed strong antibacterial activity against *Staphylococcus aureus* and *Staphylococcus saprophyticus.* Similarly, the methanolic and aqueous extracts of crude leaf powder were found to be most effective against some of the pathogenic bacterial strains.[6]

In the present study, *Bacillus cereus* KR9 produce IAA in the range of 0.9ml concentration of standard IAA in YMD broth with L- tryptophan and it was checked by Salkowski reagent. Similarly, reported that five IAA producing positive strains from rhizosphere soil. Detection of IAA was checked by the Van Urk Salkowski reagent was an important option for qualitative and semi-qualitative determination.[14] Same protocol was used and the bacterial isolates were screened for their ability to produce plant growth regulator, IAA.[8] Varying levels of IAA production were recorded. The range of IAA production in PSBs isolates with tryptophan was 57 - 288 µg/ml. while indole butyric acid was in range of 22 – 34 µg/ml.

O. sanctum nodal explants are micropropagate in MS medium with IAA, NAA, BAP and KIN showed frequency of adventitious microshoot induction and proliferation was done by 0.5ml of IAA mg/L and 3 ml of NAA mg/L the multiple shoot initiation was occurred in 0.5ml of BAP mg/L and 0.5ml of KIN mg/L in 2 weeks of time interval. Similarly[9] reported that the Maximum numbers of shoots were induced on medium

containing 0.5 mg 1-1 BA. In earlier[17] showed that the highest percentage of shoot initiation of nodal segment of *O. sanctum* was found in 10-20 days at 2 mg/L NAA. The best shoot initiation and proliferation was done by different combination of growth hormones (0.4ml of IAA mg/L + 0.5ml of BAP mg/L) and the multiple shoot initiation of *O. sanctum* was occurred in 0.2ml of NAA mg/L + 0.5ml of KIN mg/L. Similarly, reported that the Maximum number of shoots per culture of *O. sanctum* of nodal explant was recorded in MS medium containing 2.0 mg/l BAP in a mixture of 0.5 mg/l NAA.[15] Regenerated shoots of *O. sanctum* were rooted most effectively in full MS medium supplemented with 1.0 mg/l NAA.

The internodal explants were initiate in the MS media with Bacterial Supernatant with different concentrations, the highest degree of shoot proliferate at 0.2 ml IAA mg/L + 0.5 ml BAP mg/L and the best average shoot length was 2.29±0.32cm occurred within a week. Similarly, showed that the frequency of shoot induction was high in media that were supplemented with 10%CW in the presence of bacterial auxin of IAA.[7] Other reports also showed the Murashige and Skoog medium supplemented with 9.0, M 2, 4-D + 2.0, M IAA, showed maximum callus induction[19] and high range of callus induction on 1.8, M fungal IAA + 1.0, M IAA (HiMedia) showed the fungal IAA in plant cell cultures.

Protein contents of *in vivo Ocimum sanctum* leaves were identified as 120 KDa with high density of protein band. The protein range of 114 KDa of *in vitro* cultures leaves of *O. sanctum* and the protein bands in the range of 90KDa for the Microbial *in vitro O. sanctum* leaves in SDS – PADE analysis. In earlier studies of *Echinocactus grusonii* plants, *in vitro* propagated plants were taken for protein banding pattern analysis by SDS–PAGE.[10]

Ocimum sanctum was used to estimate the secondary metabolites by HPLC from *in vitro* and microbial *in vitro* callus. Eluted compounds were compared with standard graph of *O. sanctum* and the compounds were quantified with their area and their specific retention time. Similarly, reported that the Eugenol compound was eluted from *Ocimum sanctum* quantified by High Performance Liquid Chromatography.[11]

CONCLUSION

The present study concluded that, the *Ocimum sanctum* L. was chosen as a medicinal plant for micropropagate, because, it is a "Queen of Herb" and medicinal properties of *Ocimum sanctum* to role against the wide range of disease. The main aim of this study was to investigate the possible role of indole acetic acid (IAA) from bacteria to induce *in vitro* adventitious shoots in internodal explants of *Ocimum sanctum* L.

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