GC-MS Based Metabolomic Profiling of *Lactobacillus plantarum* Isolated from *Ocimum gratissimum* Rhizosphere

Abstract

L. plantarum is a known member of the lactic acid bacteria that possesses antimicrobial activities. Organic acids, hydrogen peroxide and bacteriocins have been majorly identified as the contributors to its antimicrobial activity. The present study sought to employ the GC-MS technique to further classify biological compounds that comprise the antimicrobial metabolite produced by *L. plantarum* through sub-merged fermentation. The bacterium was isolated from the rhizosphere of *O. gratissimum* and confirmed using molecular typing. Preliminary antibacterial screening of the organism was done with indicator strains isolated from urinary tract and wound surface, after which sub-merged fermentation was employed for the production of secondary metabolites for a 24 h period. The GC-MS technique was employed to identify the volatile bioactive compounds that comprise the secondary metabolite produced. The organism had significant (p<0.05) inhibition of the indicator strains when compared to the ciprofloxacin standard antibiotic. Metabolomics analyses identified Hydroxylamine, O-decyl-, 2,4-Di-tert-butylphenol, and a wide range of organic compounds mainly from the alkane, amine, carboxylic acids and phenol functional groups, as the components of its antibacterial metabolite. GC-MS based metabolomics analyses is a profitable tool for identifying key components of the antibacterial substance produced by *L. plantarum* as this will give a room for its bio-prospecting potentials as alternative and sustainable source of novel antimicrobial compounds and other beneficial medications used by humans.

Key words: *Lactobacillus plantarum*, GC-MS, *Ocimum gratissimum*, antimicrobial activity.

Introduction

Rhizosphere is a narrow zone of soil surrounding roots of plants (Prashar *et al*., 2013), known to be of high microbial diversity ranging from bacteria, fungi to actinomycetes (Mendes *et al*., 2013). This is basically as a result of increased nutrient supply from photosynthesis which releases different organic compounds to the root and encourage the existence and proliferation of some of these microbes at the rhizosphere (Odelade and Babalola, 2019).

The genus, *Ocimum*, has been classified as a power plant genus (Pandey *et al*., 2021) because of its many species known to have several medicinal advantages. This genus of plants has been in the fore-front of alternative provisions to antibiotic therapy as well as for the production of new antimicrobial compounds from the African and Asian context (Tiwari *et al*., 2021). In addition to its much medical and industrial importance, its environmental importance is not left out as seen from the diversity of medically important microorganisms it harbors in its rhizosphere. *Ocimum gratissimum* and *Ocimum basilicum* are the two most popular species of the plant that have been extensively reported for their richness in rhizosphere organisms that are of importance with regards to antimicrobial activity and bioremediation respectively (Prapagadee and Khonsue 2015; Tiwari *et al*., 2021; Pandey *et*

al., 2021). Some bacteria identified to be associated with Rhizospheres of *Ocimum* plants include *Achromobater* spp., *Serratia* spp., *Ochrabactrum* spp., *Bacillus* spp.; basically isolated from *Ocimum gratissimum* and *Ocimum basilicum*. Due to the desire for new antimicrobials to be produced as a means of regulating antibiotic resistance threat currently facing the world, researchers have concentrated efforts in the rhizosphere habitat as a rich source of microorganisms with antibacterial, antifungal and even anti-helminthic activities.

Lactobacillus is a genus of bacteria reported extensively for their antibacterial and probiotic potentials. Species of this genus are facultatively anaerobic, Gram positive, catalase negative rods; found in different habitats such as fermented foods, vagina, colon and other habitats that possess some degree of anaerobiosis, and also degree of mesophilic and thermophilic temperatures (Bhattacharya *et al*., 2022; Raman *et al*., 2022). The rhizosphere being an anaerobic habitat and also a rich habitat for organisms with antimicrobial potentials is thought to possibly harbor some species of *Lactobacillus*. This study thus had the objectives of isolating *Lactobacillus* species with antibacterial activity from rhizosphere of *Ocimum gratissimum*; production of antibacterial compounds from the choice isolate using sub-merged fermentation; and identifying the volatile bioactive compounds that constitute its antibacterial properties using gas chromatography-mass spectrometric analyses.

Methods

Isolation of Microorganism from Rhizosphere

Isolation of *Lactobacillus* was performed by serial dilution and plating technique using nutrient agar and De Mann Rogosa and Sharpe agar (MRS) medium. One gram of each soil sample was suspended in 9 ml sterile water in a test tube. Ten fold serial dilution was done up to fifth dilution, and then 1 ml was collected from each 10^{-2} dilution and plated on the agar media. The nutrient agar plates were incubated for 24 h aerobically and anaerobically at 25^{o}C (Atsede and Fassil, 2018).

Isolation and Identification of the Test Bacterial Isolates from Urine and Wound Samples

Bacterial isolates were isolated from urine samples according to the method described by Tanzina *et al*. (2016). Clean-catch midstream morning urine specimen was collected using sterile wide mouth glass containers. Until laboratory analysis, the samples were kept cooled in a refrigerator. The time between sample collection and the sample analysis did not exceed one hour. Using sterile wire loops, 0.01 ml urine sample was then plated onto blood agar and MacConkey agar plates, incubated aerobically at 37℃ for 24hrs. This was used for the isolation of *E. coli*, *Klebsiella* and *Staphylococcus aureus* from urine samples.

Pseudomonas aeruginosa was isolated from wound sites using method described by MacFadden (2000). With sterile swab sticks, wound swabs were taken carefully from the site

of infection and placed in tubes containing normal saline to maintain the swab wet during transferring to laboratory. Each specimen was inoculated on cetrimide agar plates supplemented with 1% glycerol and allowed to incubate for 24 h at 28°C.

Biochemical Characterization for *Lactobacillus* species

Isolates were characterized using Gram Staining, catalase, motility and sugar fermentation characteristics as described by Cheesbrough (2006).

Molecular Identification

Isolates were characterized using 16srDNA molecular typing

Production of Antibacterial Metabolites from the Bacterial Isolates

About 50 ml of the fermentation medium containing the following (g/L); L-glutamic acid 5.0; KH_2PO_4 0.5; KH_2PO_4 0.5; $MgSO_4.7H_2O$ 0.2; $MnSO_4.H_2O$ 0.01; NaCl 0.01; $FeSO_4.7H_2O$ 0.01; $CuSO_4.7H_2O$ 0.01 ; CaCl.2H2O 0.015; Glucose 10; and PH 7); and 1% glucose, was prepared in 250 ml Erlen-Meyer flask and sterilized. Stock culture of organisms was prepared by inoculation of a loop-full of each bacterium in 10 ml MRS broth. Stock cultures were incubated for 24 h, after which 5 ml of the stock culture was transferred into the sterile 50 ml fermentation medium, incubated in a rotary shaker at 120 rpm for 24 h, anaerobically at 28°C. Broth cultures were filtered firstly with whatman No.1 filter paper and secondly with nitrocelloluse membrane (0.45 μm pore diameter) after incubation (Sethi *et al*., 2013). The filtrate was chilled and kept for gas chromatography and mass spectrometry analyses.

Extraction and Identification of Bioactive Compounds Present in the Produced Antibacterial Metabolites using Gas Chromatography and Mass Spectrometry.

This was analyzed according to AOAC 1990

Preparation of standard

A 10 ul aliquot of accu standard was injected in the chromatography and the retention time compared with retention time of standard.

Extraction of bioactive compounds from samples

1ml of filtered residue was dissolved in 50ml of chloroform, transferred to a 100ml volumetric flask and diluted to the mark. Most of the chloroform was evaporated at room temperature, 1 ml of the reagent {20 vol% benzene and 55 vol% methanol} was added, Sealed and heated at 40^0c water bath for 10 minutes. After heating, the organic sample was extracted with hexane and water, so that the final mixture of the reagent, hexane and water, is in proportion of 1:1:1 (i.e., 1ml each of hexane and water was added to the reaction mixture). The mixture was shaked vigorously by hand for 2min. A stable emulsion that was formed was broken by centrifugation. Then about half of the top hexane phase was transferred to a

small test tube for injection. Proper care was taken in ensuring that only the organic layer was removed. And injection was not done directly from the reaction vial because of the risk of injecting water. Water can ruin the GC column.

Gas Chromatographic conditions for bioactive compound determination

The final extracts were analyzed by **Gas Chromatograph-Buck M910 scientific gas chromatography equipped with Electron capture detector** that allowed the detection of contaminants even at trace level concentrations (in the lower µg/g and µg/kg range) from the matrix to which other detectors do not respond. The GC conditions used for the analysis were capillary column HP 88 capillary column (100m x 0.25µm film thickness,) CA, USA

The injector and detector temperature were set at 250 ºC and 290ºC respectively. The oven temperature was programmed as follows: 110 ºC held for 10 min, ramp at 10 ºC/ min to 200 ºC, held for 5min, and finally ramp at 10 ºC/ min to 320 ºC. Helium was used as carrier gas at a flow rate of 1.0 mL/ min and detector make-up gas of 29 Ml min-1. The injection volume of the GC was 8.0 µL. The total run time for a sample was 48 min.

Quantification of bioactive compound residues.

The residue levels of the bioactive compounds were quantitatively determined by the external standard method using peak area. Measurement was carried out within the linear range of the detector. The peak areas whose retention times coincided with the standards were extrapolated on their corresponding calibration curves to obtain the concentration.

Statistical Analyses

Statistical Analyses was done using GraphPad Prism version 8. Mean values were compared using one way Analyses of Variance (ANOVA), at 95% confidence interval.

Results

Isolation, Characterization and Antibacterial Screening of *Lactobacillus plantarum* from Rhizosphere Samples

Lactobacillus sp. was characterized as shown in Table 1 and was confirmed using molecular typing as *Lactobacillus plantarum*. The isolate was screened for antibacterial activity using the organisms isolated from urine and wound sites as shown in Figure 1. This organism significantly ($p<0.05$) inhibited the growth of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli* when compared to the ciprofloxacin standard (Figure 1).

Production and GC-MS Evaluation of Antibacterial Substances from *Lactobacillus plantarum*

Gas chromatography and mass spectrometry analyses showed forty-two bioactive compounds present in the metabolites. The top five bioactive compounds that possibly contributed predominantly in the antibacterial activities of *Lactobacillus plantarum* are shown in Figure 2 and Table 2.

Table 1: Biochemical Characteristics of *Lactobacillus plantarum*

Biochemical Tests	**Results**
Gram stain	+
Cell morphology	rod
Catalase reaction	-
Motility	-
Glucose fermentation	+
Gas from glucose	-
Lactose fermentation	+
Sucrose fermentation	+
Galactose fermentation	+
Probable Organism	*Lactobacillus* sp.

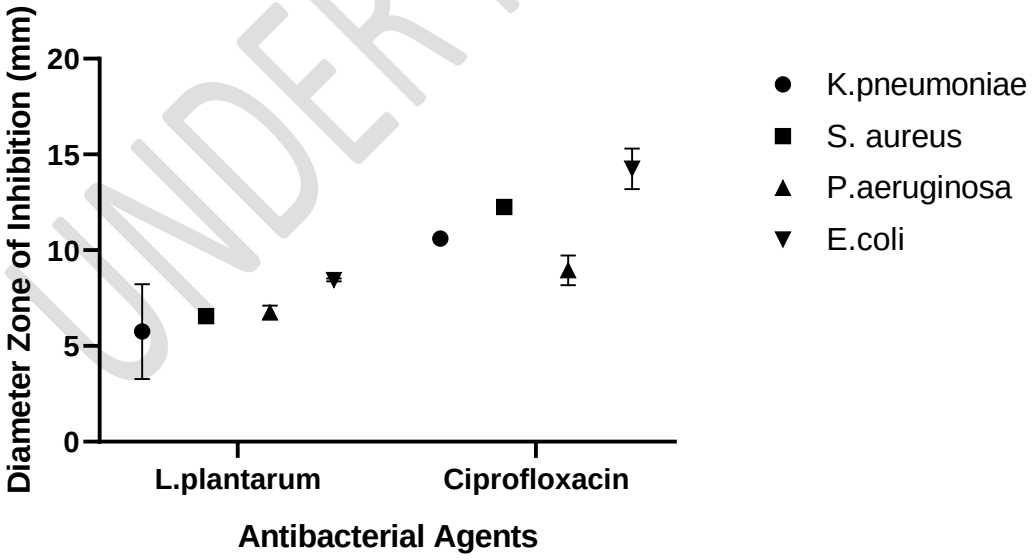

Figure 1: Antibiotic Screening of *L. plantarum* against Selected Organisms.

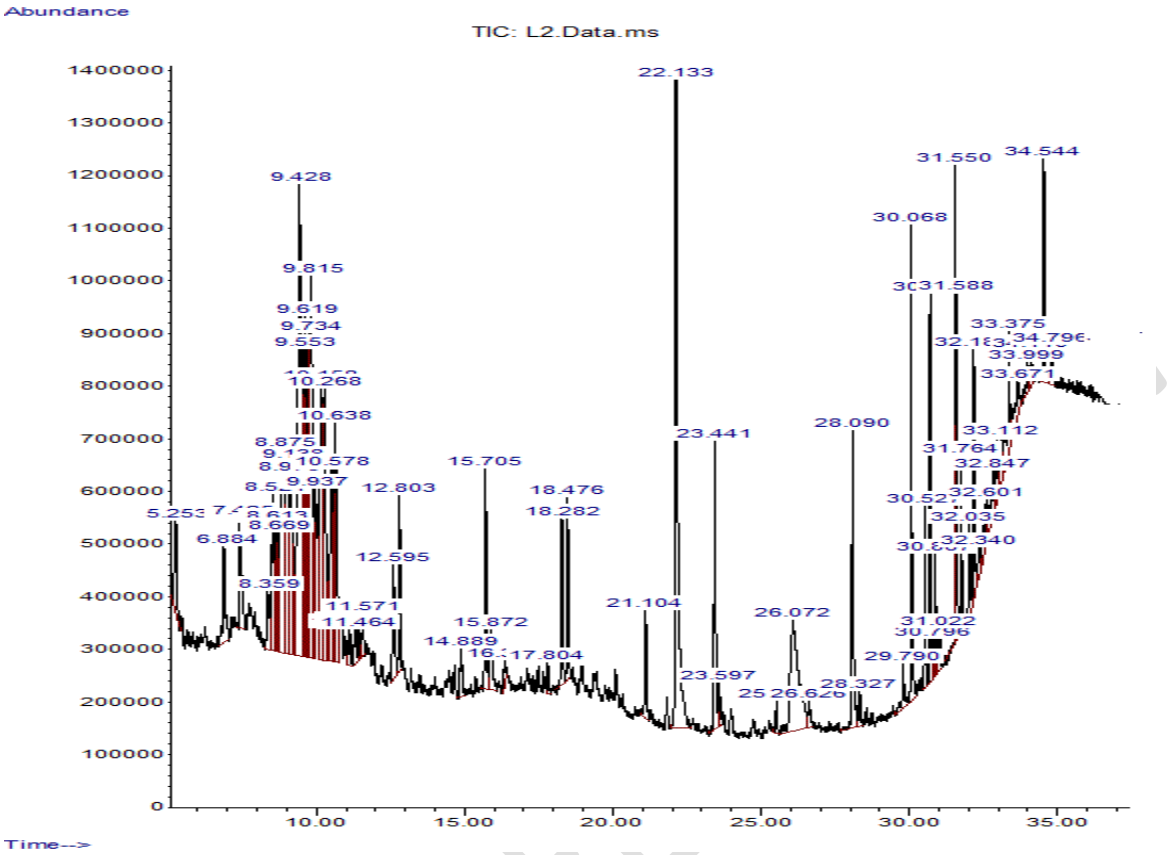

Figure 2: Gas Chromatogram showing Elution Peaks of Identified Bioactive Compounds Produced by *L. plantarum*.

Table 2: Predominant Metabolites Produced by *Lactobacillus plantarum*

Peak numbers	Retention time	Area%	Metabolite names
11	9.428	8.19	a) Docosane, 2,21-dimethyl- b) Hydroxylamine, O-decyl- c) Carbonic acid, decyl vinyl ester
36	22.133	7.74	a) 2,4-Di-tert-butylphenol b) Phenol, 3,5-bis(1,1-dimethylethyl)
17	10.158	5.09	a) 1-Decanol, 2-hexyl- b) Carbonic acid, nonyl prop-1-en-2-yl ester c) Hexadecane, 7-methyl-
15	9.815	4.89	a) Undecane b) Decane, 2-methyl- c) Heptadecane, 2,6,10,14-tetramethyl
40	26.072	4.36	a) Cyclohexene, 6-butyl-1-nitro- b) 8-Heptadecyne, 1-bromo-

	c) 9-Oxabicyclo [6.1.0]nonane

Discussion

L. plantarum is a well–researched lactic acid bacterium known for its various antimicrobial characteristics both bacteriocin-mediated and non-bacteriocin mediated. Various food sources are known to be normal habitats where this microorganism can be isolated from. This study however, isolated this microorganism as part of the rhizosphere organisms for *Ocimum gratissimum*, commonly known as 'scent leaf' in Nigeria.

Antibacterial screening of this organism against urinary tract pathogens and surface wound pathogens using the agar well diffusion assay of the cell-free supernatant showed significant ($p<0.05$) inhibition of *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli*. This finding partly corresponds with the reports of De Giani *et al*. (2019) and Hu *et al*. (2019). Lin and Pan (2019) used similar indicator strains used in this study and had a similar report as that found in this finding. However, a striking finding from this study that differs from theirs is that *S. aureus* strain they used was susceptible to both the cell-free supernatant and bacteriocin tested from *L. plantarum*, but in this study, *S. aureus* showed resistance to the cell-free supernatant of *L. plantarum*. Comparing the inhibitory capacity of *L. plantarum* with that of ciprofloxacin, it was observed that their inhibition differed significantly ($p<0.05$) with *E. coli* and *P. aeruginosa* in this study. According to the review report of Dinev *et al*. (2017), *L. plantarum* inhibitory properties are basically organic acid -, hydrogen peroxide-, bacteriocin-mediated, which constitute volatile and non-volatile inhibitory properties.

A host of some scientists (Lin and Pan, 2019; De Giani *et al*., 2019; Chaudhary *et al*., 2020; Ladha and Jeevaratnam, 2020; Huang *et al*., 2022) have put in efforts in this regard to identify further the bioactive components in antimicrobial metabolites produced by *L. plantarum*. Some of the probing methods they have employed include the Fourier Transform-Infra Red (FT-IR) analyses (Ladha and Jeevaratnam, 2020), Electro spray ionization (ESI) mass spectrometry (De Giani *et al*., 2019), High performance liquid chromatography (HPLC) analyses (Lin and Pan, 2017), Nuclear magnetic resonance (NMR) profiling (Lin and Pan, 2017) and also the GC-MS analyses. The present study carried out untargeted metabolomics of *L. plantarum* volatile inhibitory substances using the GC-MS analyses. A total of 64 bioactive compounds were identified from culture supernatant in this study while Huang *et al*. (2022) identified 43 metabolites, and Chaudhary *et al*. (2020) identified 10 compounds from *L. plantarum* DB2 also using GC-MS. They also used GC-MS to analyze metabolites produced by *L. plantarum* alongside other lactic acid bacteria, and their metabolite findings partly correspond with the metabolites identified in this study. Dawwam *et al*. (2022) identified 22 bioactive antimicrobial compounds from *L. plantarum* using GC-MS, some of their reported compounds partly corresponded with those found in this study. Assessing the top five predominant metabolites identified and shown in Table 2, Hydroxylamine, O-decyl- is known to be toxigenic to some bacteria by having mutagenic effects on them as reported by Zhang *et al*. (2022); 2,4-Di-tert-butylphenol has been reported by Vinati Organics (2021) as a secondary metabolite with a wide range of toxicity to various microorganisms; Hexadecane, 7-methyl- is a petroleum compound reported for its antimicrobial activity by Yu *et al*. (2013). Other hydrocarbon compounds

identified by the GC-MS analysis possess antimicrobial activities and thus contribute to the antibacterial activity expressed by *L. plantarum* used in this study.

Conclusion

This metabolomics study of *L. plantarum* has shown that a wide range of organic compounds, mainly from the alkane, amine, carboxylic acids and phenol functional groups predominantly make up the volatile antibacterial compounds produced by this vastly studied lactic acid bacteria which also doubles as a probiotic microorganism.

REFERENCES

Atsede M., Fassil A. Isolation and Screening of Antibiotic ProducingActinomycetes from Rhizosphere and Agricultural Soils (2018). *African Journal of Biotechnology*, **17**(22):700-715.

Bhattacharya D., Nanda PK., Pateiro M., Lorenzo J.M., Dhar P., Das, A.K. Lactic Acid Bacteria and Bacteriocins: Novel Biotechnological Approach for Biopreservation of Meat and Meat Products (2022). *Microorganisms*, **10**:2058.

Chaudhary A., Verma K., Saharan, B.S. A GC-MS Based Metabolic Profiling of Probiotic Lactic Acid Bacteria Isolated from Traditional Food Products (2020). *Journal of Pure and Applied Microbiology*, **14**(1): 657-672.

Cheesbrough M. District Laboratory Practice in Tropical Countries-Part2 (2006). Cambridge University Press, New York, 2nd edn. pp: 47-55.

Dawwam G., Saber II., Yassin MH., Ibrahim, HF. Analysis of Different Bioactive Compounds Conferring Antimicrobial Activity from *Lactobacillus plantarum* and *Lactobacillus acidophilus* with Gas Chromatography-Mass Spectrometry (GC-MS) (2022). *Egyptian Academic Journal of Biological Sciences*, **14**(1): 1-10.

De-Giani A., Bovio F., Forcella M., Fusi P., Sello G., Di-Genaro, P. Identification of A Bacteriocin-like Compound from *Lactobacillus plantarum* with Antimicrobial Activity and Effects on Normal and Carcinogenic Human Intestinal Cells (2019). *AMB Express*, **9**:88.

Dinev T., Beev G., Tzanova M., Denev S., Dermendzhieva D., Stoyanova, A. Antimicrobial Activity of *Lactobacillus plantarum* against Pathogenic and Spoilage Organisms: A Review (2017). *Bulgarian Journal of Veterinary Medicine*, DOI: 10.15547/bjvm.1084

Hu C., Ren L., Zhou Y., Ye, B. Characterization of Antimictobial Activity of Three *Lactobacillus plantarum* Strains Isolated from Chinese Traditional Dairy Food (2019). *Food Science and Nutrition*, **7**: 1997-2005.

Huang P., Yu L., Tian F., Zhao J., Zhang H., Chen W., Zhai, Q. Untargeted Metabolomics Revealed the Key Metabolites in Milk Fermented with Starter Cultures Containing *Lactobacillus plantarum* CCFM8610 (2022). *LWT-Food Science and Technology*, **165**:113768.

Ladha G., Jeevaratnam K. A Novel Antibacterial Compound Produced by *Lactobacillus plantarum* LJR13 Isolated from Rumen Liquor of Goat Effectively Controls Multi-Drug Resistant Human Pathogens (2020). *Microbiological Research*, **241**: 126563.

Lin TH., Pan, TM. Characterization of an Antimicrobial Substance Produced by *Lactobacillus plantarum* NTU 102 (2019). *Journal of Microbiology, Immunology and Infection*, **52**: 409-417.

Mendes R., Garbeva P., Raaijmakers JM. The Rhizosphere Microbiome: Significance of Plant Beneficial, Plant Pathogenic, and Human Pathogenic Microorganisms (2013). *FEMS Microbiology Reviews*, 37:634-663.

Odelade KA., Babalola OO. Bacteria, Fungi and Archae Domains in Rhizospheric Soil and their Effects in Enhancing Agricultural Productivity (2019). *International Journal of Environmental Research and Public Health*, 16: 3873.

Pandey V., Swami RK., Narula A. Harnessing the Potential of Roots of Traditional Power Plant: *Ocimum* (2021). *Frontiers in Plant Science*, **12**: 765024.

Prapagdee B., Khonsue N. Bacterial Assisted Cadmium Phytoremediation by *Ocimum gratissimum* L. in Polluted Agricultural Soil: A Field Trial Experiment (2015). *International Journal of Environmental Science and Technology*, **12**: 3843-3852.

Prashar P., Kapoor N., Sachdeva S. Rhizosphere: Its Structure, Bacterial Diversity and Significance (2013). *Reviews on Environmental Science and Biotechnology*, doi: 10.1007/s11157-013-9317-z.

Raman J., Kim JS., Choi KR., Eun H., Yang D., Ko YJ., Kim, S.J. Application of Lactic Acid Bacteria (LAB) in Sustainable Agriculture: Advantages and Limitations (2022). *International Journal of Molecular Sciences*, **23**:7784.

Tiwari S., Pandey R., Gross, A. Identification of Rhizospheric Microorganisms that Manages Root Knot Nematode and Improve Oil Yield in Sweet Basil (*Ocimum basilicum* L.) (2021). *Agronomy*, **11**: 570.

Yu C., Yao J., Cai M., Wang F., Masakorala K., Liu H., Blake R.E., Doni S., Ceccanti, B. Functional Gene Expression of Oil-Degrading Bacteria Resistant to Hexadecane Toxicity (2013). *Chemosphere*, **93**:1424-1429.

Zhang M., He T., Chen M., Wu, Q. Ammonium and Hydroxylamine can be Preferentially Removed During Simultaneous Nitrification and Denitrification by *Pseudomonas taiwanensis* EN-F2 (2022). *Bioresource Technology*, **350**: 126912.