



# **Bioactive Metabolites of Nodule Associated Microbes for Enhanced Drought Tolerance and Biocontrol Control Activity in Horsegram**

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## **Authors' contributions**

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

## **Article Information**

DOI: 10.9734/IJPSS/2022/v34i1831074

## **Open Peer Review History:**

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: <https://www.sdiarticle5.com/review-history/86774>

**Original Research Article**

**Received 02 March 2022**

**Accepted 11 May 2022**

**Published 11 May 2022**

## **ABSTRACT**

In the present study, the potential bioactive compounds detected in the ethyl acetate microbial extract of root nodules of horsegram were determined using the gas chromatography-mass spectroscopy (GC-MS). The bioactive metabolites of nodule associated microbes (NAM) revealed the existence of several soluble metabolites which includes phthalic acid, butyl hex-3-yl ester, 2,4-Di-tert-butylphenol, Dodecyl acrylate, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro, 1-Nonadecene, octadecane, 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester, hexadecanoic acid, methyl ester, dibutyl phthalate, diisooctyl phthalate, 1-docosene, heptadecane, 9-hexyl, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl), n-Tetracosanol-1, ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'(phenylmethyl)-, (5'à,10à), eicosanal, ethanol, 2-(9-octadecenyloxy), E-15-heptadecenal, octatriacontyl pentafluoropropionate, etc.,. Endophytic bacteria produces bioactive substances that provides resistance against phytopathogens as well as nutrient solubilization during prolonged drought periods in order to overcome biotic and abiotic stresses. Several antibacterial, antifungal, antiviral and antioxidant properties were brought under spotlight to realise the beneficial aspects of nodule associated microbes of horsegram. Understanding the roles of metabolites would enrich the crop growth under stressed environment by promoting the eco-friendly agriculture practices.

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*Keywords: Metabolites; bioactive compounds; antioxidants; antimicrobials; rootnodules; horsegram.*

## 1. INTRODUCTION

Secondary metabolites are low molecular weight organic compound involved in communication between microbial communities, host plants and their resourceful niches unearthing the potential of many defense molecules, growth hormones and stress tolerants and protectants [1]. They can counteract the abiotic stresses caused due to adverse environments by acting as osmoprotectants, ROS scavengers and antioxidants [2]. Studies on endophytes revealed the functional genes for the synthesis of soluble metabolites that stimulates the growth and productivity of crops against biotic and abiotic stresses and thus maintain a symbiotic relationship with plants [3]. Changing the orientation of roots, stomatal regulation, adjustment of osmotic potential and maintaining the homeostasis condition of ROS mechanisms are some of the adaptive techniques by plants against drought conditions [4]. The presence of functional and regulatory genes involved in signalling pathways exhibits an indirect mechanism to tackle drought conditions imposed on plants by regulating oxygen-related metabolisms, activating the expression of osmoregulatory compounds, namely proline and betaine [5].

Bringing forth the massive production of exopolysaccharides, upregulating the gene expression for 1- aminocyclopropane-1-carboxylate (ACC) deaminase, induction of stress tolerant metabolites the enhanced drought tolerance by plant growth growth promoting rhizobacteria [6]. Low molecular organic compounds interactions of various beneficial microorganisms and host plants differ based on phenotypic characteristics, developmental stages of plants lead to the colonization of diverse microbes in below ground and above ground plants [7].

This study focuses on the stress-tolerant properties, osmoregulatory factors, and defensive roles of microbial metabolites extracted from the nodule-associated microbes of horse gram. Understanding the crop-specific metabolic functions may give rise to a significant formulation of metabolites for legume crop production and protection by reducing the use of threatful chemical fertilizers.

## 2. MATERIALS AND METHODS

### 2.1 Preparation of Microbial Culture and Extraction Process

A total of 10 nodule-associated microbes, namely HGR1, HGB1, HGB2, HGB3, HGB4, HGB5, HGB6, HGB7, HGY1, HGY2 were isolated from horse gram crop. Microbial cultures were grown in Erlenmeyer flask containing Luria-Bertani broth (bacteriological tryptone 10g, yeast extract 5g, NaCl 10g, distilled water 1000ml) incubated in rotary shaker at 110 rpm at 28 °C until it reached the late log phase for metabolite production. The inoculated broth is filtered and extracted using ethyl acetate in a separating funnel [8]. The concentrated metabolites extracted from the inoculated broth were dissolved in 1 mL HPLC grade methanol and used for GC-MS analysis (Perkin Elmer®, USA).

### 2.2 Characterization of Soluble Metabolites through GC-MS Analysis

The identification of soluble metabolites present in the different nodule-associated microbial isolates was analyzed with a GC-MS (Gas chromatograph Clarus®, 680 and Mass spectrometer Clarus® SQ8C. The GC-MS studies were performed using a 30 m X 0.32 m capillary column with a cross-band of 5% diphenyl - 95% dimethyl polysiloxane and a 70 eV ionization energy (Elite 5). The oven was set to a temperature of 40 °C for 2 minutes, followed by a 10 °C min<sup>-1</sup> run to 250 °C, which was held for 2 minutes, and the detector temperature was set to 320 °C, with the input and source lines set to 230 °C. Helium (He) was used as a carrier gas at a linear flow rate of 20 cm<sup>s-1</sup>. The scanning mode of the MS technique was chosen, with a solvent delay of 0.00 to 0.50 minutes. The GC approach was selected with a 20-minute run duration [9]. The MS detector was set to 200 °C, and the substances found were identified using a mass spectral database National Institute of Standards and Technology library (NIST).

## 3. RESULTS AND DISCUSSION

Ten nodule associated microbes, namely HGR1, HGB1, HGB2, HGB3, HGB4, HGB5, HGB6, HGB7, HGY1, HGY2 isolated from the root nodules of horse gram crop revealed the

presence of many bioactive compounds as analyzed through Gas chromatography and mass spectroscopy (Fig. 1). Several bioactive metabolites such as phthalic acid, butyl hex-3-yl ester, 2,4-Di-tert-butylphenol, Dodecyl acrylate, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro, 1-Nonadecene, octadecane, 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester, hexadecanoic acid, methyl ester, dibutyl phthalate, diisooctyl phthalate, 1-Docosene, heptadecane, 9-hexyl, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl), n-Tetracosanol-1, ergotaman-3',6',18-trione, 9,10-

dihydro-12'-hydroxy-2'-methyl-5'(phenylmethyl)-, (5'α,10α), eicosanal, ethanol, 2-(9-octadecenyl), E-15-Heptadecenal, octatriacontyl pentafluoropropionate were identified in different peak area percentages and retention time in each microbial isolates (Fig. 2). Many of the metabolites with chemical formula and retention time (Table 1) were known for renowned of being antioxidant, ROS scavenger, antibacterial, antifungal, antiviral, nematocidal, insecticidal, and drug resistance functions (Table 2).

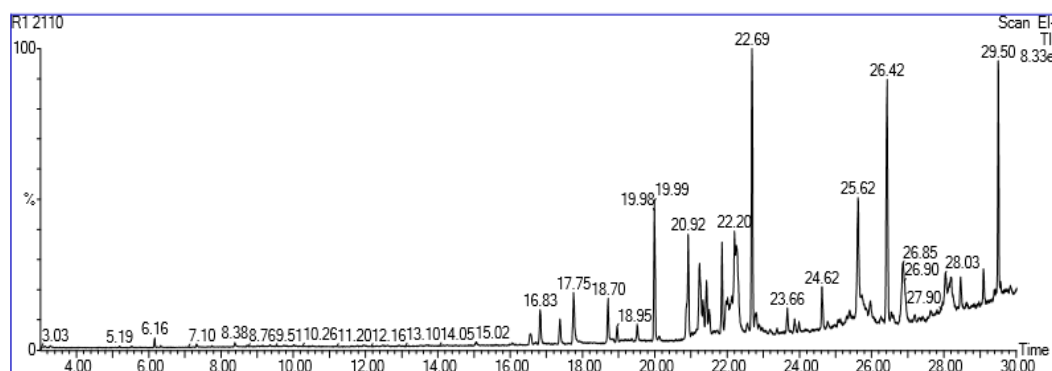


Fig. 1A. GC-MS Chromatogram of HGR1 culture extract

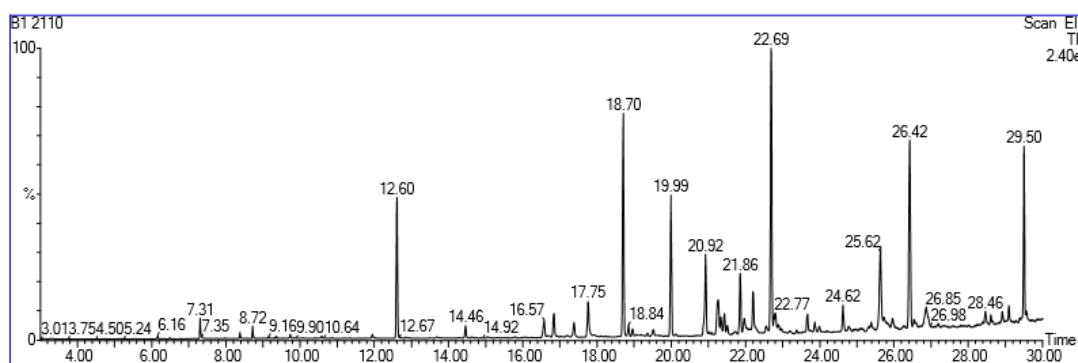


Fig. 1B. GC-MS Chromatogram of HGB1 culture extract

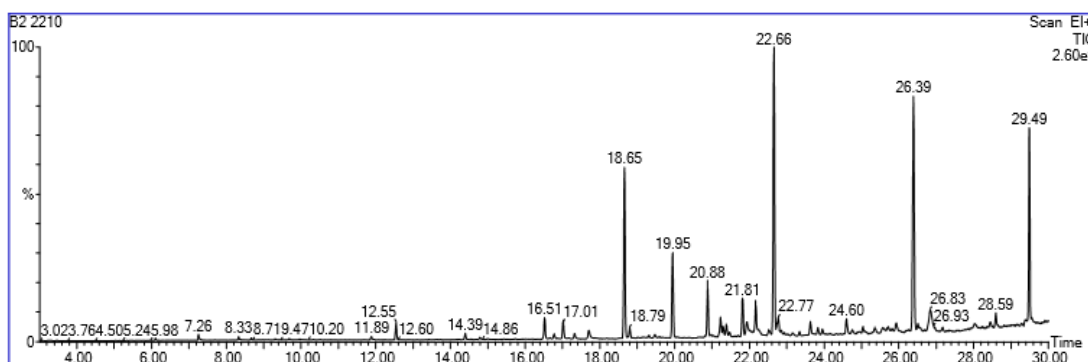


Fig. 1C. GC-MS Chromatogram of HGB2 culture extract

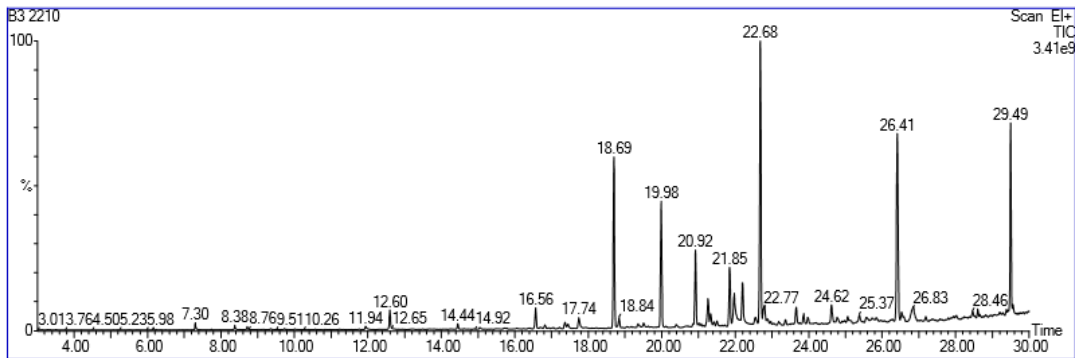


Fig. 1D. GC-MS Chromatogram of HGB3 culture extract

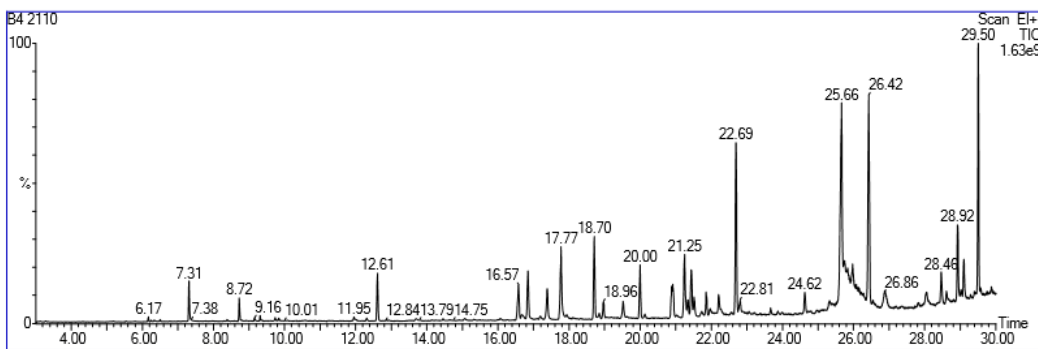


Fig. 1E. GC-MS Chromatogram of HGB4 culture extract

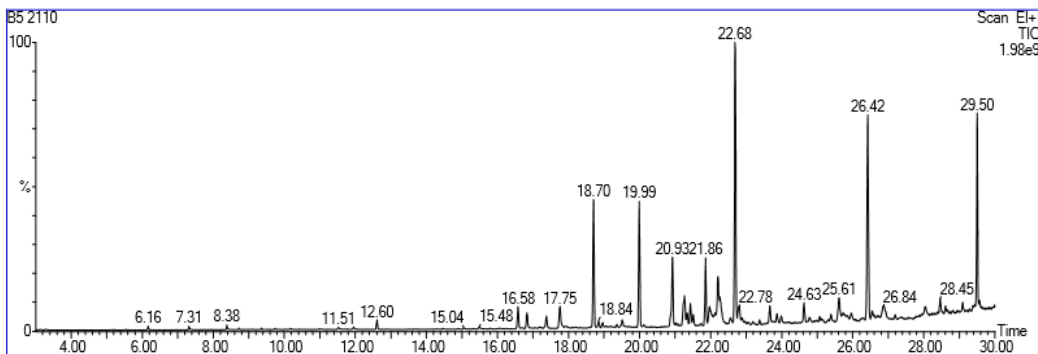


Fig. 1F. GC-MS Chromatogram of HGB5 culture extract

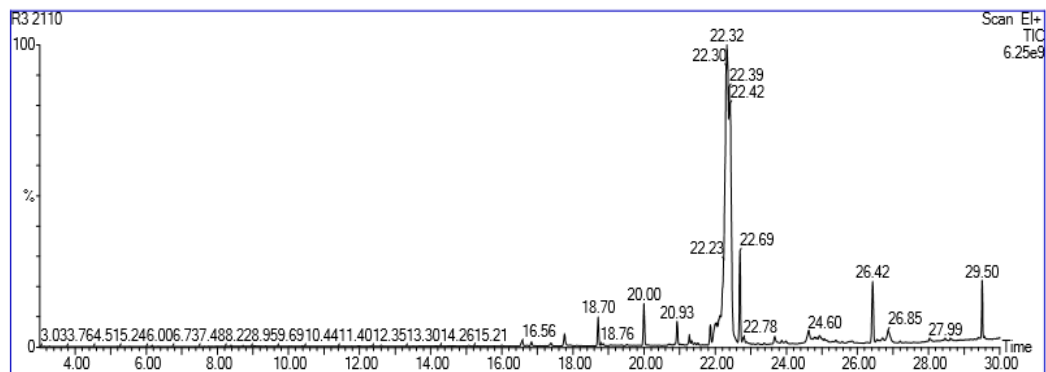


Fig. 1G. GC-MS Chromatogram of HGB6 culture extract

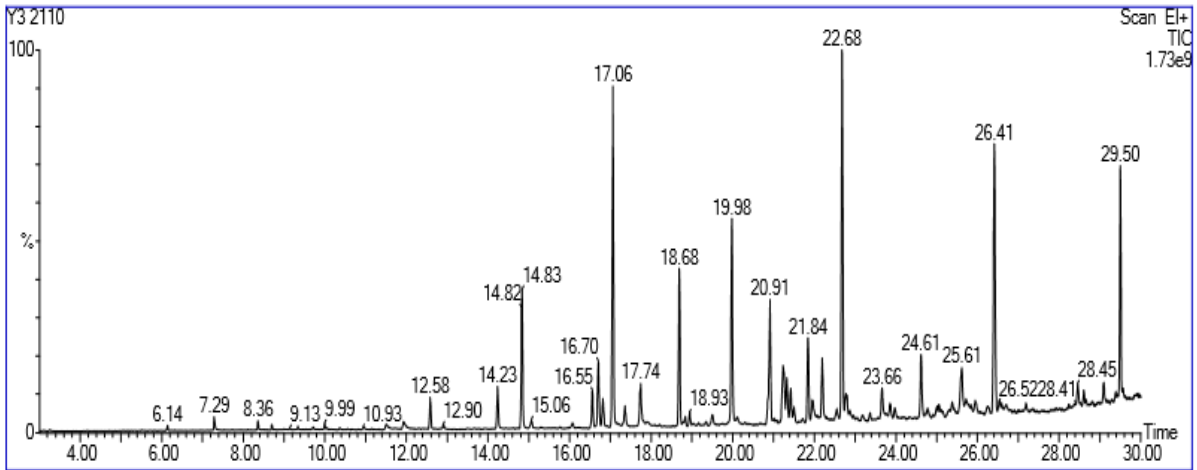


Fig. 1H. GC-MS Chromatogram of HGB7 culture extract

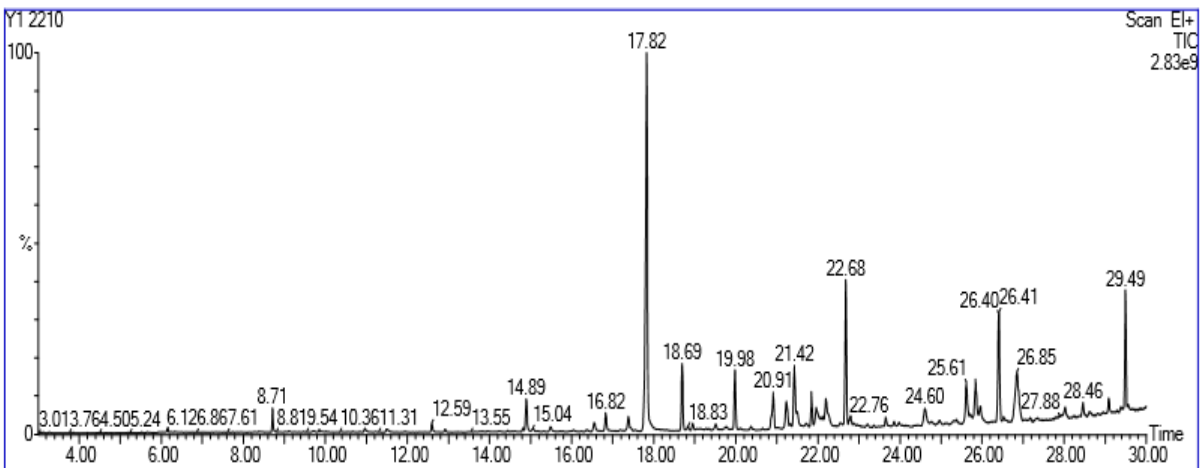


Fig. 1I. GC-MS Chromatogram of HGY1 culture extract

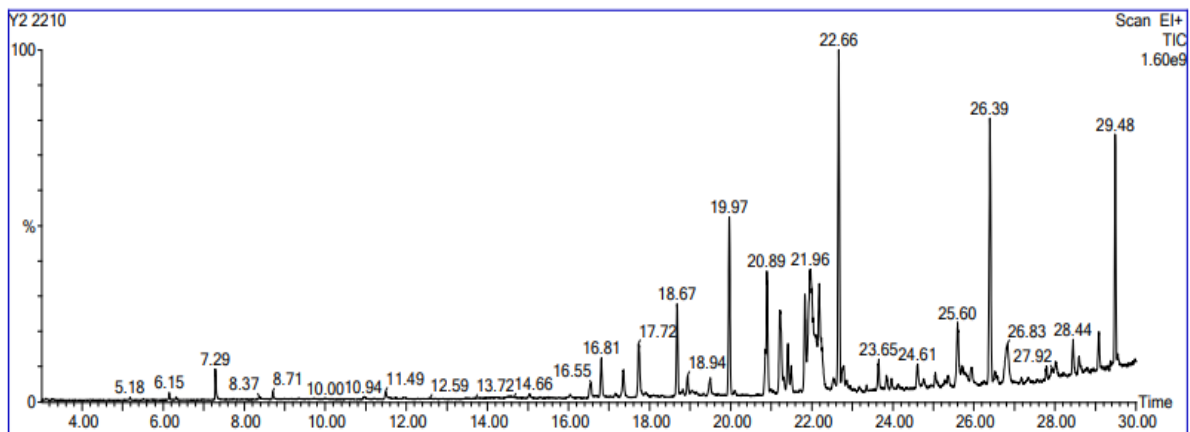
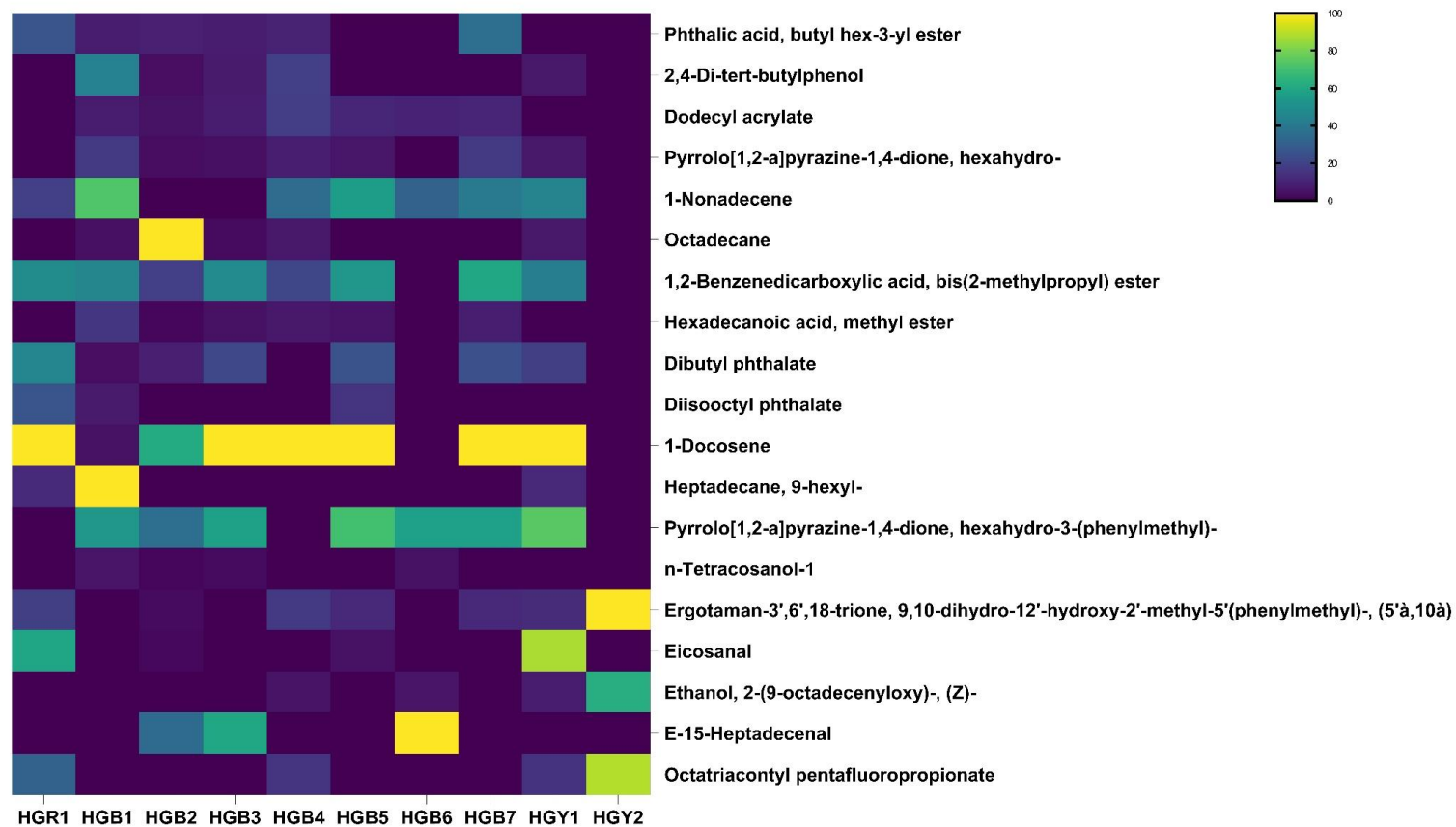


Fig. 1J. GC-MS Chromatogram of HGY2 culture extract



**Fig. 2. Heatmap representing the relative amount of soluble metabolites present in nodule associated microbes isolated from horsegram crop through GC-MS analysis**

**Table 1. Overview of characterized soluble metabolites found in the nodule-associated microbial extract of horsegram crop**

<b>Metabolites</b>	<b>Molecular formula</b>	<b>Retention time</b>
Phthalic acid, butyl hex-3-yl ester	C <sub>19</sub> H <sub>28</sub> O <sub>4</sub>	23.661
2,4-Di-tert-butylphenol	C <sub>14</sub> H <sub>22</sub> O	12.602
Dodecyl acrylate	C <sub>15</sub> H <sub>28</sub> O <sub>2</sub>	16.569
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	C <sub>10</sub> H <sub>16</sub> N <sub>2</sub> O <sub>2</sub>	17.699
1-Nonadecene	C <sub>19</sub> H <sub>38</sub>	18.705
Octadecane	C <sub>18</sub> H <sub>38</sub>	18.795
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	19.945
Hexadecanoic acid, methyl ester	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>	21.291
Dibutyl phthalate	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	22.766
Diisooctyl phthalate	C <sub>24</sub> H <sub>38</sub> O <sub>4</sub>	22.566
1-Docosene	C <sub>22</sub> H <sub>44</sub>	22.686
Heptadecane, 9-hexyl-	C <sub>23</sub> H <sub>48</sub>	22.796
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	C <sub>14</sub> H <sub>16</sub> N <sub>2</sub> O <sub>2</sub>	28.458
n-Tetracosanol-1	C <sub>24</sub> H <sub>50</sub> O	29.499
Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'(phenylmethyl)-, (5'â,10â)	C <sub>33</sub> H <sub>35</sub> N <sub>5</sub> O <sub>5</sub>	28.458
Eicosanal	C <sub>20</sub> H <sub>40</sub> O	26.868
Ethanol, 2-(9-octadecenyloxy)-, (Z)-	C <sub>20</sub> H <sub>40</sub> O <sub>2</sub>	28.594
E-15-Heptadecenal	C <sub>17</sub> H <sub>32</sub> O	18.655
Octatriacontyl pentafluoropropionate	C <sub>41</sub> H <sub>77</sub> F <sub>5</sub> O <sub>2</sub>	28.043

**Table 2. Bioactive metabolites and its properties observed in the nodule-associated cell free extract of horsegram crop**

<b>Metabolites</b>	<b>Properties</b>
Phthalic acid, butyl hex-3-yl ester	Antifungal, antiviral, antibacterial
2,4-Di-tert-butylphenol	Antioxidant and antifungal properties
Dodecyl acrylate	Antimicrobial activity
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	Scavanges ROS members
1-Nonadecene	Antibacterial, antifungal, antioxidant
Octadecane	Nematicidal activity, Antibacterial and antifungal
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	Antibacterial
Hexadecanoic acid, methyl ester	Antibacterial drug resistance
Dibutyl phthalate	Antimicrobial activity
Diisooctyl phthalate	Allelopathic, antimicrobial, insecticidal
1-Docosene	Potent antioxidant
Heptadecane, 9-hexyl-	Antifungal
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	Potential antifungal compound.
n-Tetracosanol-1	Antibacterial activity
Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'(phenylmethyl)-, (5'a,10'a)	Antimicrobial (antifungal)
Eicosanal	Antioxidant
Ethanol, 2-(9-octadecenyloxy)-, (Z)-	Antioxidant
E-15-Heptadecenal	Antibacterial compound
Octatriacontyl pentafluoropropionate	Antimicrobial effects



This is the first study to explore the bioactive compounds produced by nodule-associated microbes in horse gram crops. Plants can produce bioactive compounds through natural metabolism, but to increase the amount of low molecular weight compounds, the beneficial eco-friendly plant growth-promoting rhizobacteria are inoculated to enhance the crop growth and production simultaneously bringing down the amendment of hazardous chemical fertilizers [10]. Profiling bioactive peptides produced by rhizobacteria has impacted a visible difference in the yield of potato and maize crops grown in open fields [11]. Some of the bioactive metabolites produced by *P. aeruginosa* PM 105 exhibited antibacterial activity. Additionally, the seeds bacterized with this rhizobacteria increased the germination percentage of seeds, root length, and shoot length and enhanced the secretion of plant growth hormones, namely auxins, gibberellins, and cytokinins [12].

Normal metabolism engages the production of free radicals, but a higher concentration of free radicals leads to oxidative stress as they are highly reactive [13]. Organic metabolite 2, 4-Di-tert-butylphenol produced by *Lactococcus* sp. inhibits the oxidation of biomolecules in the presence of phenolics by neutralizing free radicals to prevent oxidative damage to plants during adverse conditions [14]. ROS production increases due to exogenous factors (environmental change, usage of drugs, harmful radiation) and endogenous factors (generation of ROS in the electron transport chain, oxidation of metabolites, mechanism of phagocytosis) thus, the presence of microbial antioxidants shields and repairs the oxidative damage by scavenging the immense amount of free radicals [15]. A bioactive compound Glycyl-L-proline extracted from the *streptomyces* MUM292 has been found to be a potential antioxidant through various bioassays such as ABTS radical scavenging Activity, superoxide anion scavenging activity, DPPH radical scavenging activity, lipid peroxidation assay, and metal-chelating activities assays [16]. In the present study, we observed [1,2,4]Oxadiazole, 5-benzyl-3-(thiophen-2-yl) [17], hexahydropyrrolizin-3-one [18], glycine, N-(2-amino-1,4-dioxonyl)-, methyl ester [19], tryptophol [20], phenethylpropanamide [21], ticlopidine [22], L-Proline, N-valeryl-, decyl ester [23], trolox [24], phorbol [25] are some of the exclusively present antioxidants extracted by ethyl acetate in horse gram crop.

To withstand biotic and abiotic stresses, endophytic microorganisms produce bioactive compounds that confer resistance against phytopathogens and also render nutrient solubilization and water uptake during extreme drought conditions [26]. Interaction between PGPM and host plant is mediated through signalling compounds that resist a wide range of pathogens as antimicrobial, antifungal, and antiviral molecules [27]. Turning down the need for toxic chemicals against plant pathogens results in an environmentally friendly approach through the production of secondary metabolites [28]. Some of the effective antibacterial bioactive compounds are 5-thiazoleethanol, 4-methyl [29], 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester [30], hexadecanoic acid, methyl ester [31], docosane [32], n-Tetracosanol-1 [33], octadecane, 3-ethyl-5-(2-ethylbutyl) [34], E-15-Heptadecenal [35], Eicosane [36], oxirane, tetradecyl [37], 1H-Carbazole, 2,3,4,9-tetrahydro [38], 1-Nonadecene [39].

Some of the antifungal activity exhibiting organic compounds are 2-Piperidinone [40], heptadecane, 9-hexyl [41], ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'(phenylmethyl)-, (5'→10à) [42], androst-4-en-3-one, 17-methoxy-, 3-methoxime [43]. It is interesting to note that several antiviral compounds such as behenic alcohol [44], tetrapentacontane, 1,54-dibromo [45], Dasycarpidan-1-methanol, acetate [46] are produced by nodule associated microbes in horse gram. Despite these concerns, numerous soluble metabolites' physiological and biological importance remains largely unexplored. This is because determining the roles of some bioactive compounds in the microbial ecology is more difficult than determining the roles of antibiotically active natural products. As a result, future studies will focus on identifying the genetic features and mechanisms involved in generating bioactive metabolites by microorganisms associated with the root nodules of horse gram crop.

#### 4. CONCLUSION

The present investigation found that GC-MS analysis of the ethyl acetate extract of ten isolates from root nodules revealed the presence of multiple bioactive constituents responsible for coping against biotic and abiotic stresses. We believe this is the first report of GC-MS analysis of root nodule extract depicting the significant role of bioactive compounds in plant growth

promotion. Successful agricultural practices would be encouraged upon formulating ecofriendly soluble metabolites responsible for beneficial effects on plant growth under extreme environments and constructing inbuilt resistance against phytopathogens.

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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