

Root Nodule-associated Plant Probiotics Modulate Growth and Drought Stress Responses in Horse Gram *Macrotyloma uniflorum* (Lam.)

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Abstract

Nodule-associated plant probiotics (NAPPs) are a representative group of plant growth-promoting microorganisms that establish a mutualistic relationship with leguminous plants. The present study aimed to unravel the NAPPs of horse gram and to evaluate its potential plant growth attributes. A total of ten NAPPs were isolated and identified, which belonged to bacterial strains such as *Pseudomonas aeruginosa* HGB1, *Pseudomonas indoloxydans* HGB2, *Acinetobacter rhizosphaerae* HGB4, *Enterobacter bugandensis* HGB5, *Klebsiella michiganensis* HGB6, and *Flavobacterium anhuiense* HGB7, and yeast strains such as *Malassezia restricta* HGY1 and HGY2, and actinobacteria (*Leucobacter aridicollis* HGB3), along with *Rhizobium* sp. HGR1. The highest levels of indole acetic acid (IAA) ($39.12 \mu\text{g ml}^{-1}$), ACC deaminase ($541.2 \text{ nmol } \alpha\text{-Ketobutyrate mg protein}^{-1} \text{ h}^{-1}$), siderophores ($93.681 \mu\text{g ml}^{-1}$), phosphorus solubilization, and potassium availability were positively correlated with the isolates HGR1, HGB1, HGB2, HGY1, and HGB5. Zinc solubilization was strongly correlated with HGR1, HGY1, and HGB1. Organic acid profiling of the strains revealed their potential to enhance nutrient solubilization and drought tolerance. *Rhizobium* sp. HGR1 and the potential nodule-associated non-rhizobial endophytes (NREs), namely *P. indoloxydans* HGB2 and *M. restricta* HGY1, were selected for their performance and non-pathogenicity. Furthermore, the interaction between *Rhizobium* sp. HGR1 and multi-trait NAPPs, HGB2 and HGY1, were evaluated under 20% PEG-induced moisture-deficit stress by root exudate profiling. Metabolite profiling revealed 28 bioactive metabolites, including steroids and their derivatives, saturated hydrocarbons, quinolones and their derivatives, pyridines and their derivatives, among others. Thus, this study highlights the significant potential of NAPPs to enhance plant growth, improve drought-stress resilience, and modulate signalling pathways, thereby contributing to the sustainable production of horse gram.

Highlights

- Nodule-associated plant probiotics (NAPPs) of horse gram exhibited higher PGP traits, including the production of IAA, Siderophores, and ACC Deaminase, as well as nutrient solubilization (P, Zn, and K).
- NAPPs profiled for organic acids revealed production of acetic acid, lactic acid, oxalic acid, butyric acid, citric acid, etc.
- Interaction of HGR1 with NAPPs HGB2 and HGY1 increased seed germination and plant growth under induced drought stress (-0.46 Mpa).
- Root-exuded metabolites, viz., stigmasterol, indole, ascorbic acid, fumaric acid, oleic acid, proline, and glucopyranoside, act as signalling cues and modulate plant growth to facilitate plant health and fitness under adverse environmental conditions.
- NAPPs can serve as a novel bio-inoculant to increase drought resilience and enhance productivity in horse gram.

Introduction

Horse gram (*Macrotyloma uniflorum* Lam.), formerly known as *Dolichos biflorus*, is an essential short-duration food legume native to tropical southern Asia (Amaral et al., 2022; Jeswani and Baldev, 1990). It is a rich source of bioactive compounds with antioxidant properties and high nutritional values (Prasad and Singh, 2015), and is widely consumed by the poorest strata of society. In addition, this protein-rich fodder is widely used as livestock feed (Prakash et al., 2008). It is grown at a wide range of temperatures (Smartt, 1985), at which other crops fail to thrive. In India, horse gram is typically cultivated in the late rainy season and low-fertility soils with fewer inputs (Witcombe et al. 2008).

A vast array of nodule-associated plant probiotics (NAPPs) establishes mutualistic relationships with legume root nodules during symbiotic nodulation. It plays a significant role in promoting plant growth by producing phytohormones, secreting root exudates, enhancing organic acid production for effective solubilization and Fe chelation, and promoting the production of signaling compounds under harsh environmental conditions (Martínez-Hidalgo and Hirsch, 2017). Exploring the studies on these plant-microbe interactions reveals the complete dependency on three factors: 1) The propensity to colonize the host, 2) The supportive environment for the establishment of a symbiosis, and 3) The presence of abundant and highly diverse colonizing microbes.

The NAPPs enhance plant growth through direct and indirect mechanisms, including growth promotion, stress tolerance, and resistance to pathogens and pests. Microbiome-mediated functions are believed to initiate mostly from the belowground parts of the plant, and are transmitted as plant-mediated signals to the above-ground compartments of floral organs. Direct effects include stimulation of comprehensive plant growth through stress reduction, modulation of aminocyclopropane-1-carboxylate (ACC) deaminase expression, and the production of plant hormones, detoxification enzymes, and osmoprotectants. Indirect mechanisms also protect crops against phytopathogens and pests through antagonism or the induction of systemic resistance (Trivedi et al., 2020). A proto-cooperative mode of interaction involving the non-rhizobial endophytes (NREs) *Bacillus subtilis* NANEB1 and *Paenibacillus taichungensis* TNEB6, along with the nodule-associated endosymbiont *Rhizobium*, has been proven to play essential roles in nodulation and nitrogen fixation in the black gram crop (Raja and Uthandi, 2019).

Recent advances on plant-microbe interactions have highlighted the significant role of indole acetic acid (IAA) production as a key signalling molecule that functions as a phyto-stimulant by interacting with the plants' signalling compounds during microbial colonization (Spaepen et al.,

2007). The inoculation of *Medicago sativa* with the IAA-overproducing *Ensifer meliloti* strain RD64 indicated the increased expression of genes related to nitrogen fixation, glucose and lipid metabolism, and abiotic stress responses by stimulating the accumulation of low-molecular-weight signalling compounds within the root nodules of the host plants (Defez et al., 2019). Beneficial microorganisms synthesize ACC deaminase, which lowers plant ethylene levels by cleaving ACC, thereby enhancing resistance to diverse environmental stresses (Glick, 2005). In addition, symbiotic rhizobia and other non-nodulating microorganisms produce siderophores that facilitate iron chelation and contribute to plant disease suppression by inducing systemic resistance (Sayyed and Patel, 2011), promoting plant growth, and ensuring successful pulse production. Consistent with these findings, our earlier studies on non-nodule-associated plant probiotics, including yeasts such as *Candida glabrata* VYP1 and *Candida tropicalis* VYW1, reported enhanced production of phytohormones and siderophores, along with increased ACC deaminase activity (Geetha Thanuja et al., 2020).

Furthermore, organic acids secreted by microorganisms boost nutrient availability for plants through mineral desorption and solubilization, thereby promoting plant growth (Grover et al., 2021). Additionally, the secretion of organic acids by microorganisms plays a crucial role in regulating developmental processes, including signalling and responses to different abiotic stresses (Khan et al., 2023; Panchal et al., 2021). The interaction between organic acids and abiotic stimuli favorably sustained plant growth, thereby improving drought tolerance and immunity (Tschaplinski et al., 2019). Notably, co-inoculation of wheat with compatible strains of *Paenibacillus* sp. strain B2 (PB2), *Arthrobacter* sp. SSM-004, and *Microbacterium* sp. SSM-001 exhibited stable, durable resistance under induced drought stress (Samain et al., 2022).

Extensive research on the horse gram has primarily dealt with the plant growth-promoting microbes capable of reducing the phytotoxicity of heavy metals, such as copper (Fatnassi et al., 2015), nickel (Edulamudi et al., 2021), chromium (Dhali et al., 2021), and pentachlorophenol (Jagadeesh et al., 2011) in polluted ecological regions. However, beyond their role in bioremediation, these plant growth-promoting microorganisms – particularly non-rhizobial endophytes have not been extensively investigated for their role in promoting plant growth. Given the widespread applications of NRE strains in enhanced plant growth and pulse production, the NAPPs of horse gram remain largely unexplored. Thus, the present study aims to isolate and characterize the potential plant growth-promoting traits exhibited by NAPPs of horse gram. In addition, the study investigates the interactions among potential NAPPs under moisture-deficit stress conditions, focusing on the production of key metabolites and the associated signalling pathways, to provide insights for the exploration of underutilized legume crops. Furthermore, the study's findings are expected to yield promising sustainable bioinputs that improve nutrient-use efficiency, enhance drought resilience, and support climate-smart cultivation of horse gram.

Materials and Methods

Sample collection

For unravelling the NAPPs, root nodules of horse gram at the flowering stage were collected from three different zones near Hosur (745 m MSL, latitude of 12.68 °N and longitude of 77.93 °E), Paiyur (490 m MSL), and Thiruvannamalai (174 m MSL) in Tamil Nadu. These northwestern and northeastern zones of Tamil Nadu were characterized as semi-arid, with mean annual rainfall of less than 900 mm and temperatures ranging from 17°C to 37°C. Plant and soil samples were collected in sterile bags and transported to the Biocatalysts Laboratory, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. The soil samples collected were analyzed for physicochemical properties (Lelago and Buraka, 2019).

Isolation of nodule-associated plant probiotics (NAPPs)

To isolate NAPPs, the collected roots were gently washed under running tap water for 10 min to remove adhering soil particles. For surface sterilization, healthy root nodules were carefully excised from the cleaned roots of each plant, immersed in 70% ethanol for 30 s, then in 0.1% HgCl₂ for 2 min, and washed 3 times with sterile water under aseptic conditions. To confirm the efficiency of the nodule sterilization process, an aliquot of 100 µL of the third (final) wash was inoculated onto R2A agar plates, which were then incubated at 28 ± 2°C. The surface-sterilized root nodules were crushed with the sterile pestle and mortar, and 100 µL of aliquots were serially diluted and inoculated on the Luria Bertani Agar (LB Agar), the Yeast Extract Mannitol Agar (YEMA), and the Yeast Extract Malt Extract (YEME). The plates were incubated at 28°C for a week to promote NAPPs growth (Dhole et al., 2016). The well-grown colonies were selected and purified on appropriate media using the streak plate method. The morphologically distinct isolated colonies were examined for taxonomic identification. Furthermore, the isolates were stored in 40% glycerol at – 80°C for validation.

Phylogenetic identification of the nodule-associated plant probiotics of horse gram

Bacterial genomic DNA was isolated by the CTAB method, and the yeast DNA was isolated by the standard cell lysis method. The genomic DNA extracted from bacteria, actinobacteria, and yeast was amplified using the universal primers 27F (5'AGAGTTTGATCCTGGCTCAG 3') and 1492R

(5' GGTTACCTTGTTACGACTT 3'), NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3'), and NL4 (5'GGTCCGTGTTTCAAGACGG3'), respectively (Lee et al., 2005) using the BIO-RAD thermal cycler (Kurtzman and Robnett, 1997). Sequence identity was assessed by performing sequence similarity searches against the GenBank database (<http://www.ncbi.nih.gov/BLAST>). The phylogenetic tree was constructed in MEGA10 to determine the close and distant relationships among the sequences.

Screening of the nodule-associated plant probiotics for their plant growth-promoting attributes

The functional attributes conferred by NAPPs in horse gram were investigated by assessing the plant growth-promoting parameters such as the production of IAA, ACC deaminase, siderophores, and organic acids, and nutrient solubilization.

IAA production

A Salkowski's colorimetric method was used with the Van Urk Salkowski reagent to determine the amounts of IAA produced by each isolate. The strains were cultured for 4 days at 28°C in LB broth. After incubation, the broth was centrifuged, and 1 mL of the supernatant was mixed with 2 mL of Salkowski's reagent (2% 0.5 FeCl₃ in a 35% HClO₄ solution) and stored in the dark. After 30 min and 120 min, the optical density (OD) was measured at 530 nm (Fu et al., 2015).

ACC deaminase activity

The cultures were grown in LB and YEME broth up to the late log phase at 30°C on an orbital shaker at 150 rpm for 24 h to measure ACC deaminase activity. The cell pellets were centrifuged and then washed with 0.1 M Tris–HCl (pH 7.6) before being suspended in the minimal salt medium with 3 mM ACC as the sole nitrogen source. The amount of α-ketobutyrate produced by enzymatic hydrolysis of ACC was measured to evaluate ACC deaminase activity in the cell-free extract (Penrose and Glick, 2003). The amount of α-ketobutyrate was measured at 540 nm, and its concentration was determined using a standard curve. The Lowry method (Lowry et al., 1951) was used to determine the protein concentration.

Siderophore production

To conduct the qualitative assay for siderophore production, the chrome azurol sulfonate (CAS) agar plate method (Schwyn and Neilands, 1987) was used. All strains were inoculated in Nutrient Broth medium for 48 h at 30°C on a rotary shaker at 120 rpm, and 0.05 mL of the resulting bacterial suspension (9×10^8 cells mL⁻¹) was spotted onto CAS-agar plates in triplicate and incubated for 5 days at 30°C. An orange halo appeared around the colonies on the CAS blue agar, indicating the production of siderophores. The ratio of the halo zone diameter or the colony diameter was assessed based on size, and the findings were represented as the mean ± standard deviation (SD).

Nutrient solubilization

Phosphorus solubilization

The ability of the strains to solubilize the inorganic form of phosphorus from tricalcium phosphate was determined by spot inoculation of strains on the NBRIP medium, and their ability for solubilization was tested at 30°C in the medium (pH 7.0) supplemented with tricalcium phosphate (Nautiyal et al., 2000). Uninoculated plates were kept as the control. After 3 days of incubation, a clear zone surrounding the colonies was observed, indicating phosphate solubilization. The solubilization index was calculated as the ratio of the halo zone diameter to the colony diameter.

Zinc solubilization

To assess the zinc solubilization potential of the strains, the Alexandrov medium supplemented with 0.1% zinc oxide (ZnO) was used. Overnight-grown single colonies were aseptically transferred to respective zinc medium plates by spot inoculation. After spot inoculation, the plates were incubated in the dark at $28 \pm 2^\circ\text{C}$ for 48 h, and a distinct halo zone formed around the cultures (Sharma et al., 2012). The efficiency of zinc solubilization (SE) was estimated by dividing the diameter of the halo zone by the colony diameter.

Potassium availability

The spot test method was used to assess the strains' potential to solubilize potassium. A loopful of 48-hour-old grown strains was spotted on the modified Aleksandrov medium supplemented with potassium aluminosilicate minerals as an insoluble source of potassium. For three days, the Plates were incubated at $28 \pm 2^\circ\text{C}$. The ability of different microbial strains to develop clear zones on media was used to detect potassium availability (Parmar and Sindhu, 2013). Uninoculated plates served as a control.

Organic acid production

The strains were grown in their respective culture broths—YEMA for *Rhizobium*, LB for bacterial strains, and YEME for yeast strains—and incubated at $28 \pm 2^\circ\text{C}$ until they reached the log phase. Log-phase cultures were harvested and centrifuged at 6000 rpm for 5 min. The GC-MS analysis was performed using the purified crude cell-free extract in a PerkinElmer GC-MS Clarus® SQ 8 Mass Spectrometer (MS) equipped with the DB-5MS (Agilent, USA) capillary standard non-polar column with dimensions of 0.25 mm OD x 0.25 μm ID x 30 m length and an FID detector (Lee et al., 2012). Autochrome software was used for data integration and analysis. As standards, an HPLC-grade organic acids kit (No. 47264 from Sigma-Aldrich, USA) was used.

Compatibility assessment and consortium preparation

The potential NAPPs were selected based on their plant growth-promoting (PGP) attributes. Non-pathogenicity of each isolate was confirmed on sheep blood agar plates (HiMedia, India; Cat. No. MP1301) by the absence of hemolytic activity. The strains selected for further experimentation were *Rhizobium* sp. HGR1, *P. indoloxydans* HGB2, and *M. restricta* HGY1. Compatibility among the three NAPPs was evaluated using standard cross-streak and co-culture assays. Strains showing no inhibitory interaction were considered compatible and will be used for consortium development (Annadurai et al., 2021).

Estimation of NAPPs-induced seed germination efficiency under moisture deficit stress

Horse gram seeds (var. Paiyur 2) obtained from the Regional Research Station, Paiyur, Krishnagiri district, Tamil Nadu, were surface sterilized in 0.1% HgCl_2 for 2 min and rinsed three times with ethanol and then five times with sterile water. After sterilization, seeds were inoculated and incubated for 1 hour with overnight-grown cultures of HGR1, HGB2, and HGY1, and with co-inoculum containing HGR1, HGB2, and HGY1. Meanwhile, horse gram seeds treated with sterile water served as a control. Inoculum-treated seeds were placed on filter paper in Petri dishes containing 0, 10, 20, and 30% polyethylene glycol (PEG 6000) corresponding to osmotic potentials of 0, -0.14 , -0.29 , and -0.46 MPa, respectively. To prevent drying, Petri plates were wrapped in Parafilm and incubated at room temperature ($28 \pm 2^\circ\text{C}$). The germination percentage was estimated after 72 h of incubation using standard methods (Khodarahmpour, 2012) as follows.

Germination % =

$$\frac{\left(\frac{\text{varvec{N}} \backslash \text{varveco} \backslash \text{varvec{f}} \backslash \text{varvecs} \backslash \text{varvece} \backslash \text{varvece} \backslash \text{varvec{d}} \backslash \text{varvecs} \backslash \text{varvec{g}} \backslash \text{varvece} \backslash \text{varvec{r}} \backslash \text{varvec{m}} \backslash \text{varvec{i}} \backslash \text{varvec{n}} \backslash \text{varveca} \backslash \text{varvect} \backslash \text{varvece} \backslash \text{varvec{d}} \backslash \text{varvec{T}} \backslash \text{varveco} \backslash \text{varvect} \backslash \text{varveca} \backslash \text{varvecl} \backslash \text{varvec{n}} \backslash \text{varveco} \backslash \text{varveco} \backslash \text{varvec{f}} \backslash \text{varvecs} \backslash \text{varvece} \backslash \text{varvece} \backslash \text{varvec{d}} \backslash \text{varvecs}}{\text{varvec{T}} \backslash \text{varveco} \backslash \text{varvect} \backslash \text{varveca} \backslash \text{varvecl} \backslash \text{varvec{n}} \backslash \text{varveco} \backslash \text{varveco} \backslash \text{varvec{f}} \backslash \text{varvecs} \backslash \text{varvece} \backslash \text{varvece} \backslash \text{varvec{d}} \backslash \text{varvecs}} \right) \times 100$$

Metabolomic profiling of root exudates

The root exudates of horse gram seedlings were collected in an experimental setup. The funnel-flask setup comprised a 500 mL Erlenmeyer flask and a 150 mm diameter funnel, which was positioned at its stem end and covered with Aluminum foil with pin-sized holes. The stem was perfectly inserted into the mouth of the flask. Glass beads were poured into the funnel through the neck, and then, acid-washed sand (240 g) was added to three-fourths of the funnel. Aluminum foil was used to cover the broader opening of the funnel, and the entire setup was autoclaved for 20 min at 121°C at 15 psi. Horse gram seeds were sown in the funnel, and to induce stress in laboratory conditions, 20% PEG 6000 was added to the modified Hoagland nutrient solution. The putative root exudate metabolites produced during the interaction between HGR1, HGB2, and HGY1 were analyzed after 10 days of seedling germination with a GC-MS (Xu et al., 2021). The bioactive molecules were identified by comparing their mass spectra with those in the NIST 08 Mass Spectral Library (National Institute of Standards and Technology). The names, molecular weights, and structures of molecules were retrieved from NIST, PubChem, and HMDB databases, respectively (Leylaie and Zafari, 2018).

Statistical analysis

The datasets were subjected to a two-way analysis of variance (ANOVA), and means were separated using Duncan's multiple-range test (DMRT) at the 0.05 significance level, using SPSS version 20. Heat maps were generated using GraphPad Prism 9, run on Windows 10 (UI Haq et al., 2019). Principal component analysis (PCA) was performed to assess differences and similarities among the variables at the 5% significance level using XLSTAT 2021. 3.1 (Bressani et al., 2020). Cytoscape software version 3.7.1 was also used to analyze metabolite data and to perform pathway enrichment analyses (He et al., 2021).

Results

The main significance of this study was to unravel the NAPPs of the underutilized crop, horse gram (*Macrotyloma uniflorum* Lam.), and its importance in plant growth and drought stress mitigation. The NAPPs isolated from horse gram nodules were identified, and the metabolites in root exudates influenced by NAPPs upon their interaction under induced drought stress were profiled.

Isolation of nodule-associated plant probiotics (NAPPs) from horse gram root nodules

A total of 10 morphologically distinct isolates, each from the nodules of a different horse gram plant, were chosen (Table 1). These strains were named HGR1, HGB1, HGB2, HGB3, HGB4, HGB5, HGB6, HGB7, HGY1, and HGY2 and tested for various growth-promoting activities, which are described below.

Identification and phylogenetic analysis of nodule-associated non-rhizobial endophytes

A total of seven bacterial isolates and one actinobacterial isolate successfully yielded the corresponding 1500-bp 16S rRNA amplification products, and their resulting sequencing data were of high quality and were therefore analyzed in NCBI databases using the BLAST tool. The majority of the isolates were identified as gammaproteobacteria, while representatives of flavobacteria and actinomycetia were also observed. The isolates identified based on 16S rRNA gene sequencing were as follows: HGR1 - *Rhizobium* sp, HGB1 - *P. aeruginosa* (Accession number: ON461479), HGB2 - *P. indoloxydans* (Accession number: ON470821), HGB3 - *Leucobacter aridicollis*, HGB4 - *A. rhizosphaerae*, HGB5 - *E. bugandensis*, HGB6 - *K. michiganensis*, and HGB7 - *F. anhuiense*. The yeast isolate HGY1 was identified as *M. restricta* based on approximately 600-bp 18S rRNA gene sequences. Approximately 1500 bp of 16S rRNA gene sequences from the microbial isolates were combined to construct phylogenetic trees using the Neighbor-joining method with 100% similarity (Fig. 1A and 1B).

Plant growth-promoting attributes

IAA production

Auxin, produced by NAPPs, is one of the most studied plant growth hormones. All strains were capable of synthesizing IAA (Fig. 2). Among them, the highest IAA production was observed in HGR1 ($39.12 \mu\text{g ml}^{-1}$), followed by HGB1 ($33.19 \mu\text{g ml}^{-1}$), HGB2 ($17.487 \mu\text{g ml}^{-1}$), and HGB5 ($13.402 \mu\text{g ml}^{-1}$), while the minimum IAA production was recorded in HGY2 ($0.977 \mu\text{g ml}^{-1}$). At a 5% error margin, HGR1, HGB1, and HGB2 had significantly higher IAA yields than HGB5 (Fig. 2A).

ACC deaminase activity

The ability of NAPPs to use ACC as a significant nitrogen source was investigated. The highest ACC deaminase activity among the evaluated nodule-associated endophytes was found in HGB5 ($473.1 \text{ nmol } \alpha\text{-Ketobutyrate mg protein}^{-1} \text{ h}^{-1}$), followed by HGB1 ($326.4 \text{ nmol } \alpha\text{-Ketobutyrate mg protein}^{-1} \text{ h}^{-1}$), HGB2 ($281.0 \text{ nmol } \alpha\text{-Ketobutyrate mg protein}^{-1} \text{ h}^{-1}$), and HGY1 ($265.8 \text{ nmol } \alpha\text{-Ketobutyrate mg protein}^{-1} \text{ h}^{-1}$). In contrast, the lowest was observed in HGY2 ($52.2 \text{ nmol } \alpha\text{-Ketobutyrate mg protein}^{-1} \text{ h}^{-1}$). In comparison, HGR1 ($541.2 \text{ nmol } \alpha\text{-Ketobutyrate mg protein}^{-1} \text{ h}^{-1}$) exhibited the highest ACC deaminase activity, resulting in the cleavage of ACC into ammonia and α -ketobutyrate (Fig. 2B).

Siderophore activity

All strains produced siderophores that suppressed plant pathogens. Among them, HGB2 produced the highest amount of siderophore ($93.681 \mu\text{g ml}^{-1}$), followed by HGR1 ($90.603 \mu\text{g ml}^{-1}$), HGB5 ($90.408 \mu\text{g ml}^{-1}$), and HGB3 ($90.181 \mu\text{g ml}^{-1}$), whereas the minimum siderophore amount was recorded in HGY2 ($42.612 \mu\text{g ml}^{-1}$) (Fig. 2C).

Phosphorus solubilization

Phosphate-solubilizing strains were qualitatively screened in the modified NBRIP agar plates supplemented with insoluble tricalcium phosphate minerals were presented in Fig. 3A. The presence of insoluble phosphate in the medium led to the development of a clear zone around colonies, which indicated the phosphate solubilization to a greater extent by the NAPPs, such as HGB2, followed by HGB1, HGY1, and HGB5, and significantly higher phosphate solubilization by HGR1 was noticed. No P solubilization efficiency was observed in HGB3, HGB4, HGB6, and HGY2 at the 95% confidence level (Fig. 3A).

Zinc solubilization

Zinc is a crucial component of various enzymes that are responsible for plant metabolism. The presence of halo zones on the Bunt and Rovira medium supplemented with 0.1% ZnO indicated zinc solubilization. Zinc solubilization by the strains HGR1 and HGB1 inoculated into the medium was significantly higher than that by other strains, such as HGY1 and HGB2, at the 95% confidence level. The P solubilization efficiency was found in HGB3, HGB4, and HGB6 (Fig. 3B).

Potassium release

Some potassium-releasing microorganisms can convert insoluble potassium (K) to soluble potassium, thereby making it available for plant uptake. The presence of halo zones on the Alexandrov medium supplemented with insoluble potassium aluminosilicate minerals indicated the

K release by the microorganisms. In this study, HGR1, along with other NAPPs such as HGY1 and HGB6, was the most efficient K-releasing strain compared with the microbial strains tested at a 5% error margin. A few strains, such as HGB3 and HGY2, were unable to release K for plant growth (Fig. 3C).

Organic acid production

GC–MS–based organic acid profiling demonstrated organic acid production by the horse-gram NAPPs, such as HGR1, HGB1, HGB2, HGB3, HGB4, HGB5, HGB6, HGB7, HGY1, and HGY2 (Fig. 4). The heatmap exhibited the significant variations in both the type of organic acid and their relative abundance produced. Acetic acid, lactic acid, isobutyric acid, malic acid, and quinic acid were among the most consistently detected metabolites across several strains. In contrast, fumaric acid, succinic acid, and benzoic acid were produced only by a few strains at lower intensities. Citric acid, malonic acid, maleic acid, ascorbic acid, and benzoic acid were produced in higher concentrations by HGR1. In contrast, butyric acid, maleic acid, and malonic acid were produced in higher concentrations by HGB1. HGB2 produced higher quantities of fumaric acid, malonic acid, and lactic acid. HGB3 produced considerable amounts of isobutyric acid, butyric acid, malic acid, and malonic acid. HGB4, which produced acetic acid and maleic acid in greater quantities. Propionic acid was produced in lower amounts by HGB5. HGB6 excessively produced ascorbic acid, succinic acid, and malonic acid. HGB7 produced higher amounts of propionic and oxalic acids. HGY1 could produce higher concentrations of lactic acid, ascorbic acid, and malic acid. HGY2 produced higher concentrations of quinic acid and oxalic acid. This distinct production pattern demonstrates NAPPs functional diversity in the synthesis of organic acids associated with nutrient solubilization and stress alleviation.

Similarities and dissimilarities of the nutrient solubilization patterns

To study the nutrient solubilization patterns, such as P, Zn, and K by the NAPPs, namely HGR1, HGB1, HGB2, HGB3, HGB4, HGB5, HGB6, HGB7, HGY1, and HGY2 and the variation in the availability of these nutrients to plants, principal component analysis (PCA) was performed for all the variables simultaneously (Fig.S1). The PCA explained a total variance of 83.04% (PC1: 48.01%, PC2: 35.03%) across NAPPs. The biplot revealed a clear grouping of strains, with HGR1, HGB2, and HGB5 positively correlated with P solubilization and K availability. HGB1 and HGY1 were positively correlated with Zinc solubilization, whereas HGB3 and HGY2 were negatively correlated with all three parameters of Zn and P solubilization and K availability.

Dimensional variations in organic acid production by the isolates

PCA of organic acids production among horse gram NAPPs showed a total variance of 55.27% (PC1: 31.12% and PC2: 24.15%). Organic acids such as butyric acid, citric acid, isobutyric acid, malonic acid, and propionic acid were the loading factors of PC1, contributing to variability. Furthermore, HGR1, HGB1, and HGB3 were positively correlated with the production of organic acids. The major components contributing to the PC2 dimension were fumaric acid, lactic acid, ascorbic acid, malic acid, and oxalic acid, which were positively correlated with HGB2, HGB7, and HGY1. HGB5, HGY2, and HGB6 were, however, negatively correlated with the production of benzoic acid, succinic acid, and quinic acid. HGB4 was positively correlated with the production of acetic acid and maleic acid at low concentrations (Fig. 5).

Based on the plant growth-promoting traits and non-pathogenic nature (confirmed through sheep blood agar plates), three NAPPs, such as *Rhizobium* sp. HGR1, *P. indoloxydans* HGB2, and *M. restricta* HGY1 were selected for further studies. These strains were compatible with one another and were formulated into a consortium at a 1:1:1 volume ratio, with a cell concentration of 10^8 CFU mL⁻¹, and evaluated for their potential role in seed germination and plant growth under induced moisture-deficit stress.

Efficiency of the biotized seed germination under induced moisture deficit stress

Surface-sterilized horse gram seeds were germinated under stress at 0%, 10%, 20%, and 30%. Our study found that primed seeds co-inoculated with *M. restricta* HGY1 could germinate even under 30% PEG-induced moisture-deficit stress. The potential germination of primed seeds was observed up to 20% PEG treatment (Table S1). At the 20% stress level, the germination percentage of co-inoculum-treated seeds was found to be higher (90%) with a vigor index of 5058, compared with that of the seeds inoculated with only HGR1 (88% and 4224), HGY1 (86% and 4110), and HGB2 (84% and 3780). In contrast, lower germination percentages (54% and 2230) were recorded in the uninoculated control under induced moisture-deficit stress (Table 2). Seeds biotized with NAPPs showed a significant increase in root and shoot length. The co-inoculum-treated seeds exhibited the highest root and shoot length of 28.8 ± 0.570 and 27.4 ± 0.057 cm, respectively, which showed a significant increase over the uninoculated control (20.9 ± 0.076 cm and 20.4 ± 0.403 cm, respectively).

Profiling of metabolites in root exudates under induced moisture deficit stress upon interaction

The analysis of root exudate metabolites produced by *Rhizobium* sp. HGR1, *P. indoloxydans* HGB2, *M. restricta* HGY1, and their interactions under induced moisture stress using the funnel method revealed the presence of 28 compounds. The major compounds included steroids and

steroid derivatives, saturated hydrocarbons, quinolones and derivatives, pyridines and derivatives, prenol lipids, piperidines, phenylpropanoids and polyketides, phenol esters, organosulfur compounds, organooxygen compounds, organohalogen compounds, organonitrogen compounds, organic acids and derivatives, organoheterocyclic compounds, naphthalenes, keto acids and derivatives, indole and derivatives, glycerolipids, flavonoids, fatty acyls, diazines, carboxylic acid and derivatives, benzenoids, benzene and substituted derivatives, alkyl halides, alkaloids and derivatives, azoles, alcohols, and polyols (Fig. 6).

Primary root exudate metabolites produced by *Rhizobium* sp. HGR1 were organoheterocyclic compounds, fatty acyls, benzene, and substituted derivatives. *P. indoloxydans* HGB2 produced higher amounts of benzene and substituted derivatives, steroid and steroid derivatives, prenol lipids, organoheterocyclic compounds, and quinolones and derivatives. The root exudates of plants inoculated with *M. restricta* HGY1 were mainly composed of organoheterocyclic compounds, fatty acyls, benzene and substituted derivatives, steroids and steroid derivatives, and prenol lipids. During a tripartite interaction, the potential NAPPs produced benzene and substituted derivatives, carboxylic acids and derivatives, organoheterocyclic compounds, fatty acyls, indoles and derivatives, steroids and steroid derivatives, and flavonoids under induced moisture stress. The biochemical pathway analysis of metabolites produced during the interaction between *Rhizobium* sp. HGR1, *P. indoloxydans* HGB2, and *M. restricta* HGY1 is shown in Fig. 7. The study of metabolic pathways in Cytoscape revealed that the key metabolic pathways, including choline metabolism, TCA cycle, glycolysis and gluconeogenesis, proline and alanine metabolism, galactose metabolism, ascorbic acid synthesis, butanoate metabolism, tryptophan metabolism, fatty acid biosynthesis, phenol metabolism, and secondary metabolite synthesis are interestingly involved in the interaction between potential NAPPs.

Discussion

The *Rhizobium*-legume interaction is a well-known symbiosis that regulates symbiotic nitrogen fixation, and the comprehensive mechanisms of root nodule formation are also well explored. In addition to *Rhizobium*, a vast array of NAPPs is found in the root nodules of leguminous plants, and together they establish a mutualistic relationship that facilitates symbiotic nodulation and promotes plant growth. Several reports on NRE have explored their potential roles in nodulation, signal exchange, and plant health promotion in legume root nodules (Geetha Thanuja et al., 2020; Isaeva et al., 2010). However, horse gram remains underutilized and underexploited, despite being a rich source of bioactive compounds that confer benefits relative to other crops, including drought resistance, the ability to germinate in poor soil conditions, and tolerance to moderate salinity. Our previous study dealt with the exploration of structural and functional diversity of bacterial communities present in the soil, rhizosphere region, root nodules, and seeds of the horse gram by using 16S rRNA high-throughput amplicon sequencing technology for a better understanding of survival strategies employed by soil and plant microbiome to upgrade the bioinoculant development strategies (Evangiline and Uthandi, 2022).

The present investigation revealed that the NAPPs isolated from horse gram root nodules were a *Rhizobium* sp. HGR1, NRE bacteria viz., *P. aeruginosa* HGB1, *P. indoloxydans* HGB2, *Acinetobacter rhizosphaerae* HGB4, *Enterobacter bugadensis* HGB5, *Klebsiella michiganensis* HGB6, *Flavobacterium anhuense* HGB7, *M. restricta* HGY1, and HGY2, and an actinobacterium *Leucobacter aridicollis* HGB3. Similarly, the nodule-associated bacterium *Paenibacillus taichungensis* TNEB6 and the yeast *Candida tropicalis* VWY1 were found and identified in root nodules of black gram (Geetha Thanuja et al., 2020; Raja and Uthandi, 2019). To the best of our knowledge, this is the first report to document the presence of yeast in horse gram root nodules and its role in promoting plant growth and drought tolerance. In the present study, NAPPs of horse gram were evaluated for their potential role in plant growth promotion by assessing the IAA and siderophore production and ACC deaminase activity. Auxins produced by plant growth-promoting microorganisms exert distinct effects on plant growth and development. Furthermore, nodule morphogenesis is regulated by auxin production stimulated by phenolic compounds, which act as signaling molecules (Ghosh et al., 2015). In the present study, all NAPPs produced significant amounts of IAA. Among them, the highest IAA production was observed in the nodule partner *Rhizobium* HGR1 ($39.12 \mu\text{g ml}^{-1}$), followed by the NRE *P. aeruginosa* HGB1 ($33.19 \mu\text{g ml}^{-1}$) and *P. indoloxydans* HGB2 ($17.487 \mu\text{g ml}^{-1}$). Similarly, several reports on the nodule microbiota indicated that IAA production by nodule-associated endophytes ranged from 70 to 80 mg g⁻¹ of dry cell weight (Fu et al., 2015; Geetha Thanuja et al., 2020; Raja and Uthandi, 2019).

Microbial siderophores have a higher affinity for Fe in Fe³⁺-containing complexes and play a critical role in suppressing populations of plant-pathogenic microorganisms. The present study reported the production of siderophores by NAPPs, among which *P. indoloxydans* HGB2 ($93.681 \mu\text{g ml}^{-1}$) showed the highest siderophore production, followed by *Rhizobium* HGR1 ($90.603 \mu\text{g ml}^{-1}$). These results suggest that co-inoculating with these efficient siderophore-producing strains can be used as a reliable approach to overcome Fe limitation and suppress pathogen proliferation by sequestering Fe³⁺ in the root zone (Ferreira et al., 2019). The hydrolysis of ACC, a direct precursor of ethylene in plants, is a significant mechanism by which microorganisms facilitate plant growth and development (Zarei et al., 2020). The present study revealed that the nodule partner *Rhizobium* HGR1 exhibited a higher ACC deaminase activity ($541.2 \text{ nmol } \alpha\text{-Ketobutyrate mg protein}^{-1} \text{ h}^{-1}$), followed by the endophytic bacteria *Enterobacter bugandensis* HGB5 ($473.1 \text{ nmol } \alpha\text{-Ketobutyrate mg protein}^{-1} \text{ h}^{-1}$) and *M. restricta* HGY1 ($265.8 \text{ nmol } \alpha\text{-Ketobutyrate mg protein}^{-1} \text{ h}^{-1}$). These findings on the ACC deaminase activity are in line with those of earlier reports on the nodule-associated endophytes, such as the bacteria *P. taichungensis* TNEB6 and *Bacillus mojavensis* PJN13 and the yeasts *Candida tropicalis*

VYW1, *Hansenula saturnus*, and *Issatchenkia occidentalis* (Geetha Thanuja et al., 2020; Raja and Uthandi, 2019; Ríos-Ruiz et al., 2019; Singh et al., 2015). Further, these results confirmed that the plants grown in the presence of the beneficial ACC deaminase-producing microbes have longer roots and shoots and are more resistant to ethylene-induced stress (Glick, 2014).

Nutrients such as phosphorus and zinc were effectively solubilized by the nodule endophytes, viz., HGR1, HGB1, HGB2, and HGY1. This solubilization efficiency was achieved by the secretion of organic acids. Organic acids produced by microorganisms, such as citric acid, oxalic acid, lactic acid, and succinic acid, lower soil pH by chelating cations bound to phosphate via their carboxyl and hydroxyl groups, thereby promoting efficient solubilization (Wei et al., 2018). Application of oxalic acid along with the phosphorus-solubilizing bacteria *Bacillus* sp. PSB16 significantly influenced rhizospheric populations of aerobic rice, thereby solubilizing immobilized P via acidification, chelation, and exchange reactions (Panhwar et al., 2013). The lower the acidity constant of organic acids, the higher the P solubilization (Zúñiga-Silgado et al., 2020). Our study showed that most of the nodule-associated strains were capable of producing organic acids with a low acidity constant, such as lactic acid, isobutyric acid, butyric acid, oxalic acid, ascorbic acid, citric acid, malic acid, malonic acid, propionic acid, and fumaric acid, which can solubilize tricalcium phosphate into soluble phosphate to facilitate plant growth. The *Rhizobium* sp. HGR1 is exceptionally capable of producing an organic acid with a high pKa, namely benzoic acid, which requires more energy to synthesize via the shikimate pathway. Conserving metabolic energy would prevent cells from secreting organic acids with high pKa values, thereby preventing solubilization. Zinc-solubilizing microbes produce various organic acids that lower pH and chelate zinc cations via acidification, and they also employ other mechanisms of solubilization, such as siderophore production, proton extrusion, and the plasma membrane redox system (Kamran et al., 2017). The amounts of organic acids secreted are predicted based on the zinc sources available to the zinc-solubilizing isolates. The findings of the present study were similar to those reported for efficient zinc solubilization via the production of organic acids, namely lactic, malonic, and malic acids, by *B. aryabhatai*, *Pseudomonas taiwanensis*, and *Bacillus* sp. PAN- TM1 (Vidyashree et al., 2018). Potassium availability to crops improves root and shoot elongation and yield-related characteristics through the acidolysis mechanism employed by potassium-releasing bacteria, which release significant amounts of organic acids that break down insoluble potassium into an active biomineral form (Ahmad et al., 2016).

In addition to nutrient solubilization, the organic acids produced by NAPPs mitigate drought stress (Khan et al., 2020; Kleiber et al., 2024; Panchal et al., 2021). Kleiber et al. (2024) reported that organic acids treatment in lettuce enhanced drought tolerance by bolstering photosynthetic efficiency and strengthening oxidative defence mechanisms. Several organic acids, such as fumaric, malic, citric, malonic, ascorbic, and acetic acids, have been implicated in osmotic adjustments, ROS scavenging, and maintenance of membrane stability under water deficit conditions (Guo et al., 2018; Rahman et al., 2021; Tahjib-UI-Arif et al., 2021; Utsumi et al., 2019).

Furthermore, acetic acid enhances drought tolerance in plants by activating ABA biosynthesis and signaling, reducing stomatal conductance and transpiration, and mitigating ROS damage through the upregulation of key antioxidant enzymes (Rahman et al., 2021; Utsumi et al., 2019). Our results provide evidence that the organic acids produced by all ten NAPPs can promote plant growth by enhancing mechanisms involved in solubilization and by conferring drought-stress tolerance. Furthermore, the coexistence of NREs and *Rhizobium* had improving effects on the communication with the host plant in terms of colonization, elongation of infection threads, nodulation, nitrogen fixation, and extension of host range in legumes (Lu et al., 2017). PCA analysis proved strain-specific nutrient-solubilizing potential. Thus, the best-performing horse gram strains—HGR1, HGB2, and HGY1—emerge as metabolically compatible options for improving stress resilience and nutrient-use efficiency. Furthermore, the improved seed germination, vigor index, and seedling growth observed in NAPPs-biotized horse gram under induced moisture deficit stress (20% PEG 6000) exhibited the synergistic role of *Rhizobium* sp. HGR1, *P. indoloxydans* HGB2, and *M. restricta* HGY1 in imparting early-stage drought tolerance, consistent with reports that microbial priming boosts stress resilience and seedling vigor (Priyadharshini et al., 2023; Khatri et al., 2020).

To assess the potential role of organic acid-producing NAPPs, root exudate metabolites were profiled under induced drought stress. The metabolomic study of root exudates revealed that most of the heterocyclic compounds secreted upon interaction with NAPPs, namely *Rhizobium* sp. HGR1, with *Pseudomonas indoloxydans* HGB2, *Malassezia restricta* HGY1, and their co-inoculum (HGR1 + HGB2 + HGY1) under induced moisture-deficit stress, exhibits antibacterial, antifungal, and herbicidal activities (Saini et al., 2013). The quinolone-based quorum-sensing system in *Pseudomonas* plays an essential role in the production of virulence factors. Interestingly, the *Pseudomonas* quinolone signal (PQS) acts as a siderophore by scavenging iron, storing it in the cell membrane, and transporting it into cells with iron deficiency (Lin et al., 2018). Compounds belonging to the group of fatty acyls and prenol lipids with lipid origin are thought to be involved in signaling mechanisms of the plant-microbe and microbe-microbe interactions for effective colonization (Macabuhay et al., 2022). Indole and its derivatives have been shown to regulate virulence by activating quorum-sensing molecules (QSMs) (Lee and Lee, 2010).

The accumulation of flavonoids scavenges the free radicals and promotes plant growth under water-deficient (drought) conditions. In addition, derivatives of flavanone interact with phytohormone pathways by inhibiting auxin biosynthesis, thereby promoting drought tolerance (Aslam et al., 2022). Several metabolites released from seeds are associated with the tricarboxylic acid cycle, which is involved in the synthesis of sugars, amino acids, and lipids. Consistent with this, amino acids and lipids strongly influence the plant's resistance to severe drought stress through interactions between microbes and the host plant (Wang et al., 2022).

The present study identified most pyrimidine derivatives with significant radical-scavenging activity due to the presence of electron-donating substituents on the pyrimidine nucleus. The highest antioxidant scavenging activity of this particular compound relates to the electron with the lowest density in the outermost ring of pyrimidine derivatives (Nair et al., 2022). The most crucial metabolite discovered during the interaction between the *C. tropicalis* VYW1 and the *Rhizobium* sp. VRE1 was a glucopyranoside. It serves a specific function as a recognition receptor, implying that its ability to bind diverse sugar structures and activate lectin during interactions with potential NAPPs has been documented (Barre et al., 2001; Geetha Thanuja et al., 2020). In turn, lectin acts as a glue for saccharide receptors in the interaction between root hair tips and legume nodulating bacteria. Amino acid biosynthesis and metabolism upregulate the expression of ion-uptake genes by increasing membrane permeability, adjusting osmotic potential, and maintaining homeostasis during drought. A study supported the enhancement of the amino acid synthesis pathway under drought stress, in association with nodule development, during the *Rhizobium*-legume symbiosis (Liu et al., 2022). Our study highlights the roles of NREs in enhancing plant growth attributes, including the production of plant growth-promoting hormones, nutrient solubilization, and drought tolerance in horse gram associated with *Rhizobium* sp., under induced moisture-deficit stress. In conclusion, the adoption of sustainable agricultural practices would be enhanced by improving bioinoculant formulations, incorporating eco-friendly metabolites that promote plant growth in extreme environments, and developing built-in resistance to phytopathogens.

Conclusion

The present study characterized the NAPPs of horse gram and identified strains that promote plant growth and establishment under both normal and stress conditions. These strains may contribute to lateral root formation, increased root and shoot elongation, antioxidant accumulation for scavenging free radicals, nutrient acquisition, and mitigation of drought-induced signaling imbalances through the production of organic acids. Besides, the profiling of root exudate metabolites revealed that the interaction between the *Rhizobium* sp. HGR1 and NAPPs (*P. indoloxydans* HGB2 and *M. restricta* HGY1) within the nodules increased nodulation efficiency by producing signaling molecules. Additionally, they produced several metabolites that promote plant growth and enhance resilience to adverse environmental conditions. Further investigations into seed priming with co-inocula of NAPPs under greenhouse and open-field conditions may pave the way for the development of new bio-inoculants that enhance plant growth, fitness, and stress tolerance.

Declarations

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Author contributions

SE: Data curation, Methodology, formal analysis, Writing- Original draft, SU: Conceptualization, Funding acquisition, Project administration, investigation, supervision, Writing-review and editing; NS: Data curation, formal analysis, software, validation, Writing-review and editing. All authors read and approved the final manuscript.

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Data availability

All data generated or analysed during this study are included in this manuscript. The nucleotide sequence data from this study have been deposited in the NCBI GenBank database under the accession numbers ON461479 and ON470821.

Ethics approval and consent to participate

Not applicable

Consent to publish

Not applicable

Competing interests

The authors declare no competing interest.

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Tables

Table 1

Gram staining, cell shape, and morphological characteristics of the colony (shape, size, color, edge, and elevation) of nodule-associated plant probiotics of horse gram

Isolate name	Shape	Colour	Elevation	Edge	Gram Staining
HGR1	Circular	Creamy white	Convex	Entire	Negative
HGB1	Circular	Blue-Green	Raised	Undulate	Negative
HGB2	Irregular	Greenish Blue	Flat	Irregular	Negative
HGB3	Circular	Whitish Brown	Raised	Smooth	Positive
HGB4	Circular	White	Convex	Entire	Negative
HGB5	Circular	Pale Yellow	Raised	Entire	Negative
HGB6	Circular	Light Yellow	Convex	Entire	Negative
HGB7	Circular	Yellow	Raised	Smooth	Negative
HGY1	Irregular	White, Opaque	Raised	Undulate	-
HGY2	Circular	Creamy White	Convex	Entire	-

Table 2

Seed biotization of NAPPs induced Germination and vigour index of horse gram under moisture-deficient (20% PEG 6000) conditions.

NAPPs	Germination %	Root length	Shoot length	Vigor Index
Control	54	(20.9 ± 0.403) ^d	(20.4 ± 0.213) ^d	2230
HGR1	88	(24.1 ± 0.125) ^b	(23.9 ± 0.41) ^b	4224
HGB2	84	(22.8 ± 0.0.143) ^c	(22.2 ± 0.08) ^c	3780
HGY1	86	(24.2 ± 0.088) ^b	(23.6 ± 0.111) ^b	4110
Co-inoculum	90	(28.8 ± 0.0.570) ^a	(27.4 ± 0.057) ^a	5058

Control – Absolute control treatment without any inoculants; HGR1-*Rhizobium* sp. HGR1; HGB2- *P. indoloxydans* HGB2; HGY1- *M. restricta* HGY1; Consortium-HGR1+HGB2+HGY1. Values are mean ± (standard error) (n=5) and values with the same letter in each column differ substantially on the observation day, as indicated by DMRT ($p \leq 0.05$).

Figures

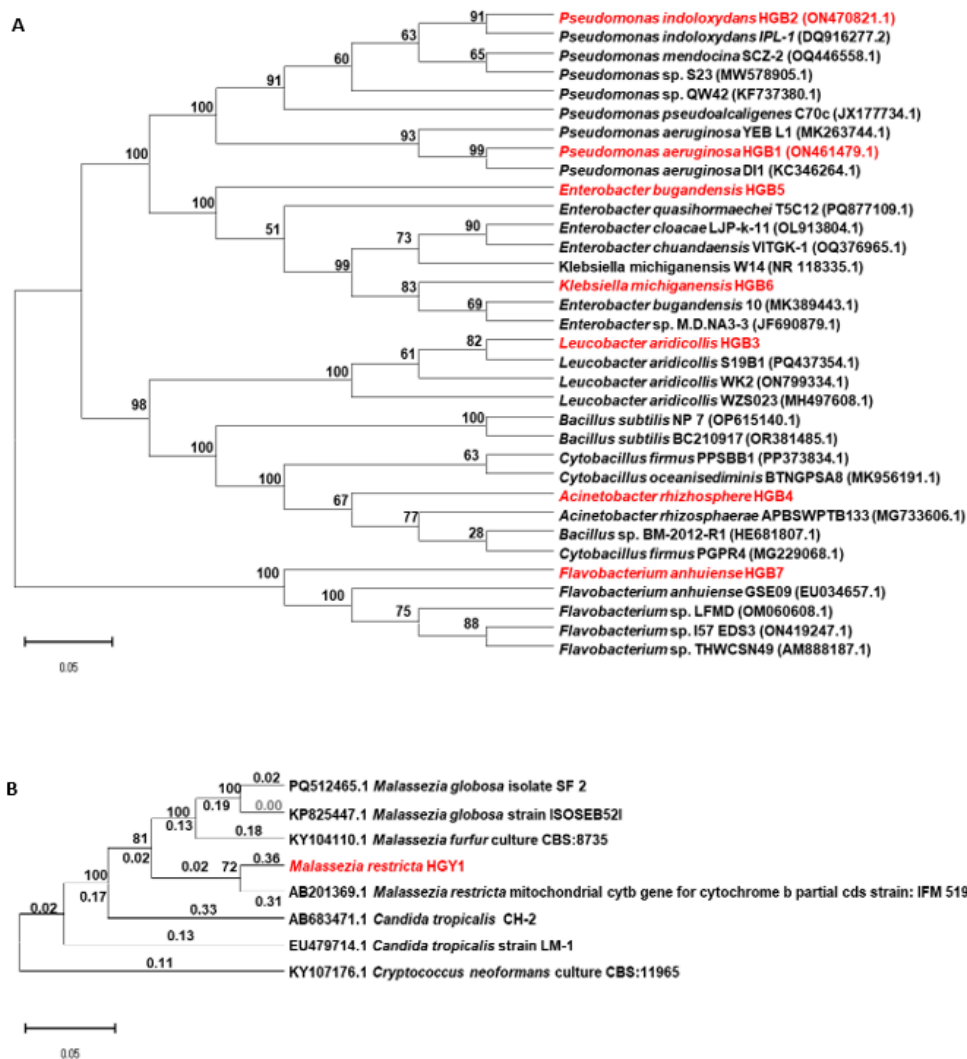


Figure 1

Phylogenetic analysis of 16S rRNA sequences using the reference sequences from NCBI

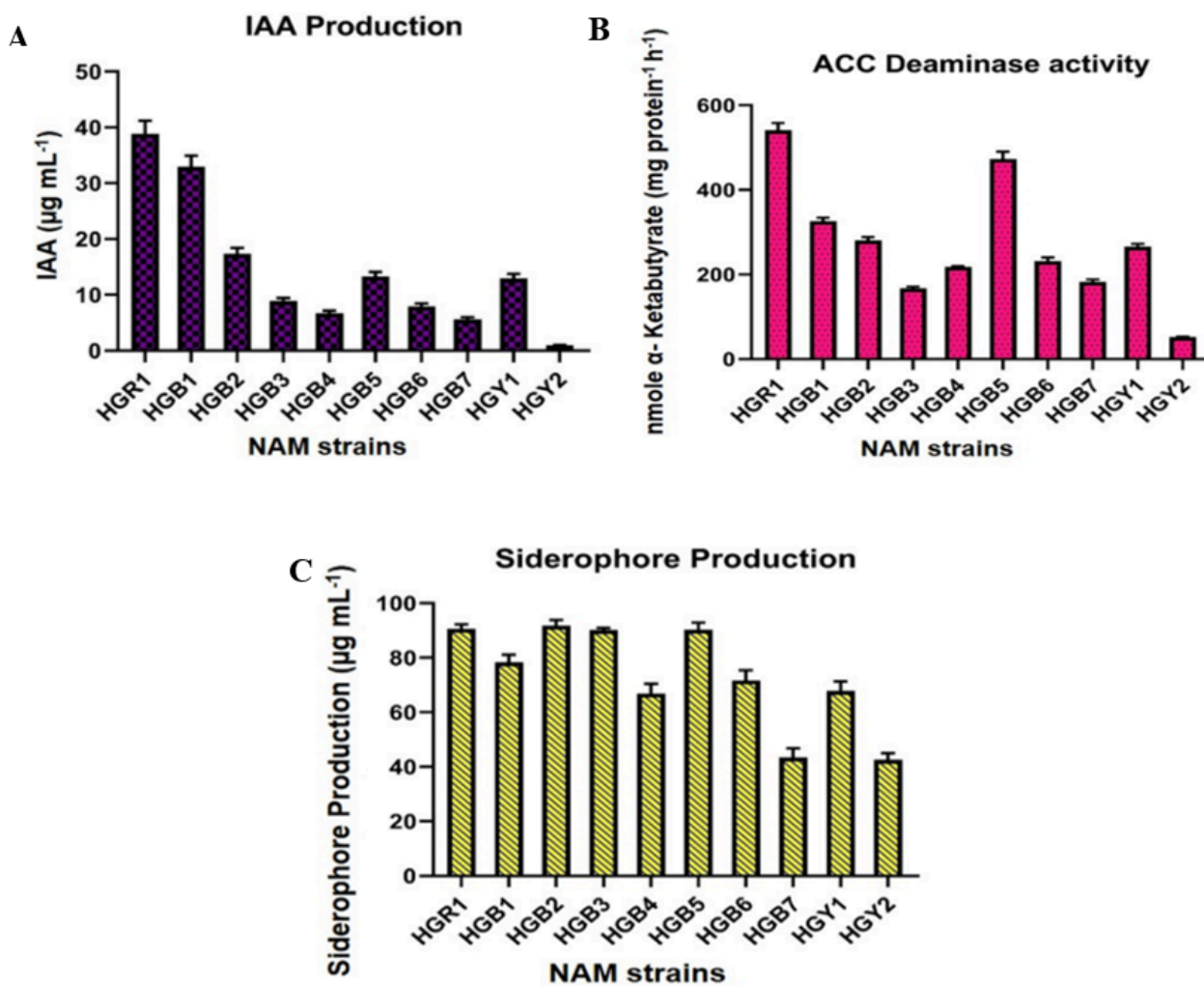


Figure 2

Plant growth-promoting traits of the nodule-associated plant probiotics (NAPPs) of horse gram. A) IAA production ($\mu\text{g mL}^{-1}$), B) ACC deaminase activity ($\text{n mol } \alpha\text{-Ketobutyrate mg protein}^{-1} \text{h}^{-1}$), and C) Siderophore production ($\mu\text{g mL}^{-1}$). Data represent the mean $\pm$ standard error of three replications ($n = 3$).

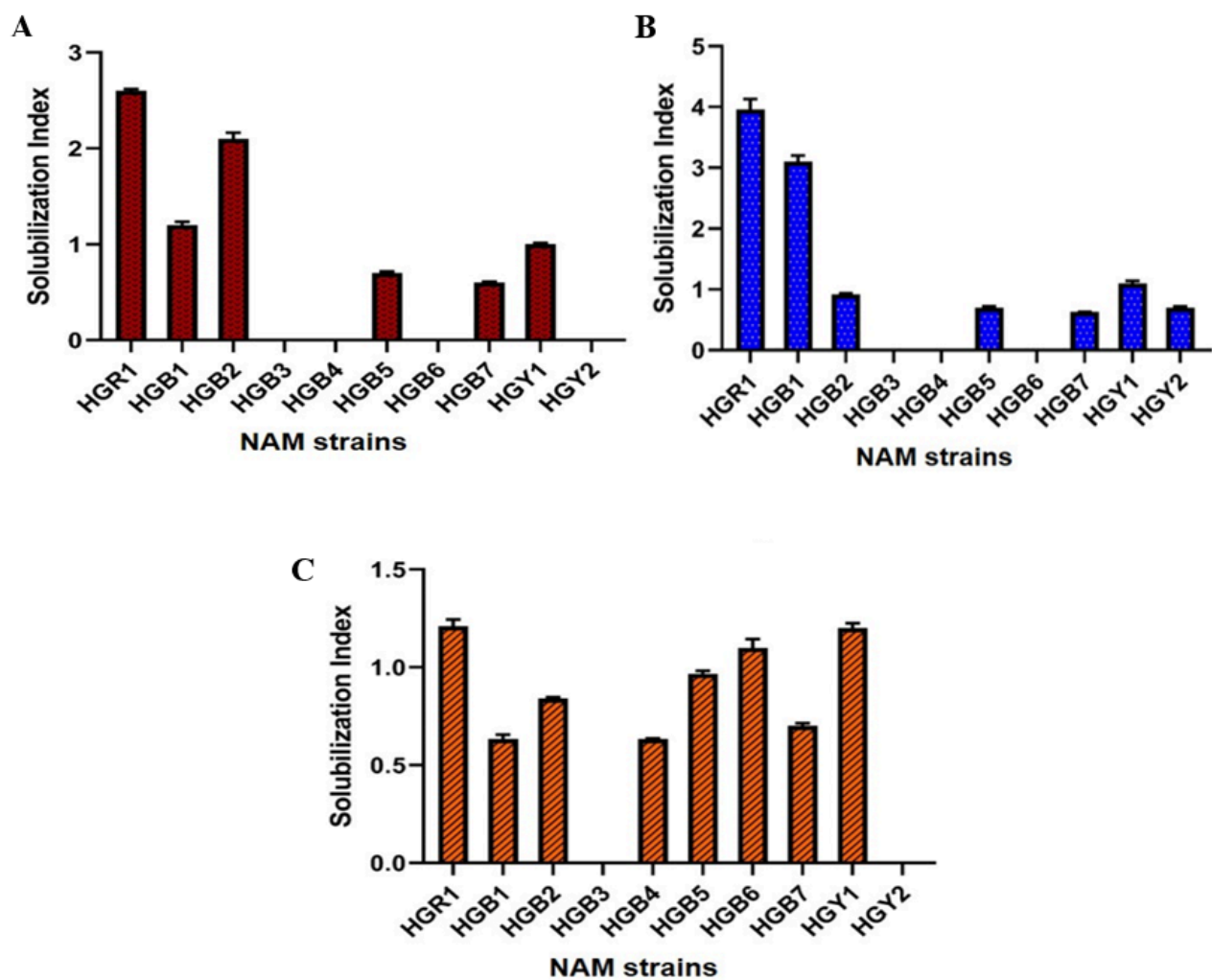


Figure 3

Nutrient solubilization efficiency by the nodule-associated plant probiotics (NAPPs) of horse gram; A) Phosphorus solubilization, B) Zinc solubilization, and C) Potassium release. Data in the graph represent the mean $\pm$ standard error of three replications ($n = 3$).

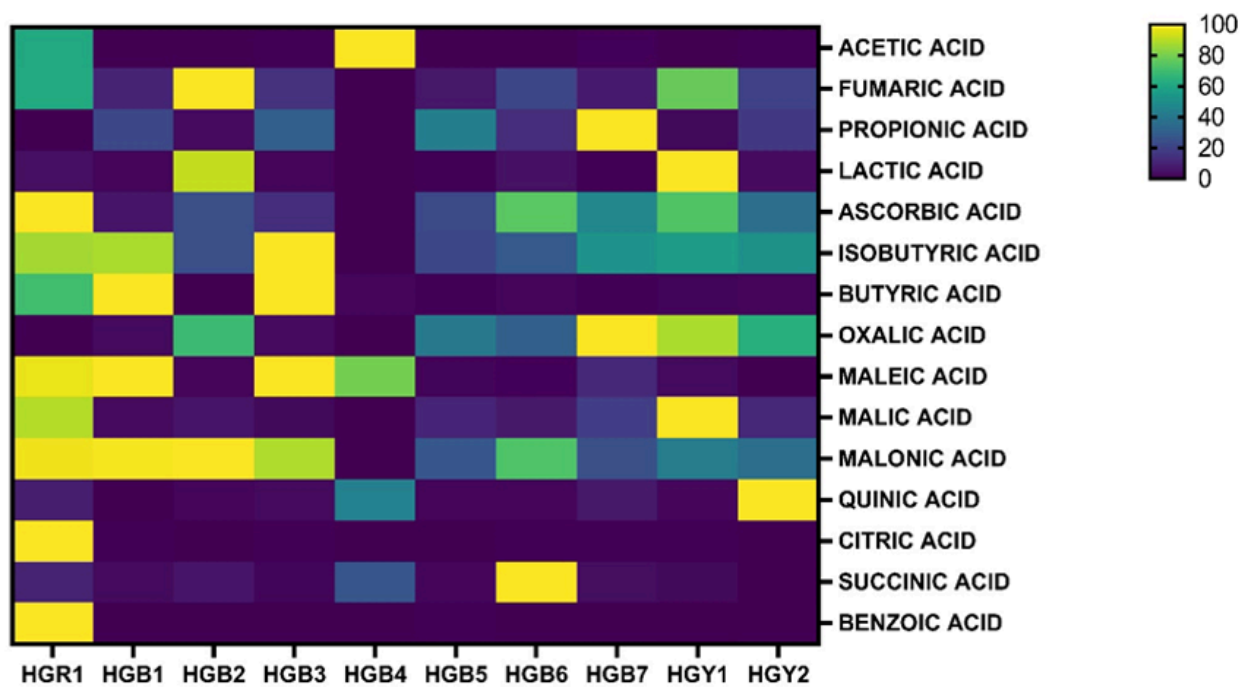


Figure 4
Heat map depicting the total amount of each organic acid (ppm) produced by nodule-associated plant probiotics (NAPPs) of horse gram

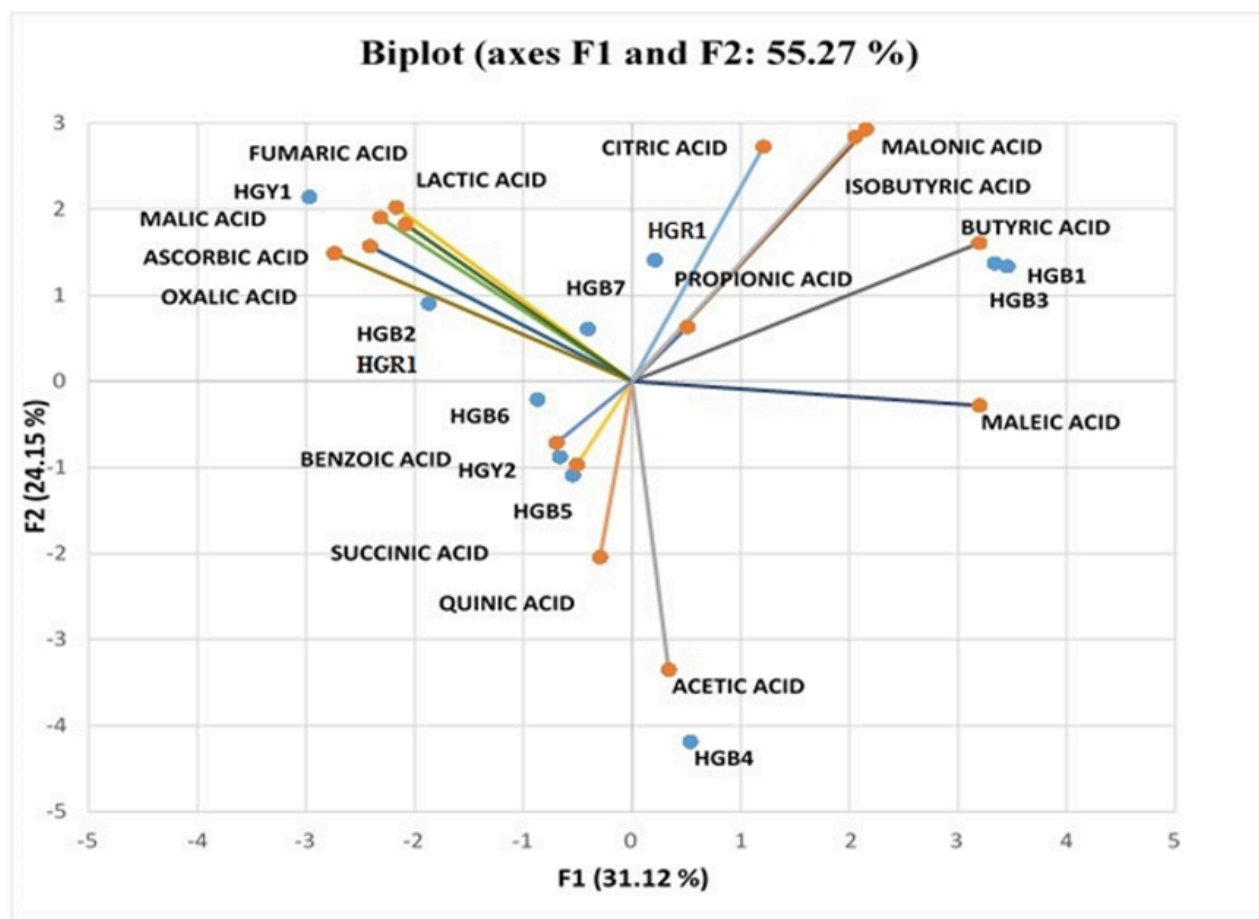


Figure 5

Biplot of principal component analysis (PCA) for the production of organic acids by the nodule-associated plant probiotics (NAPPs).

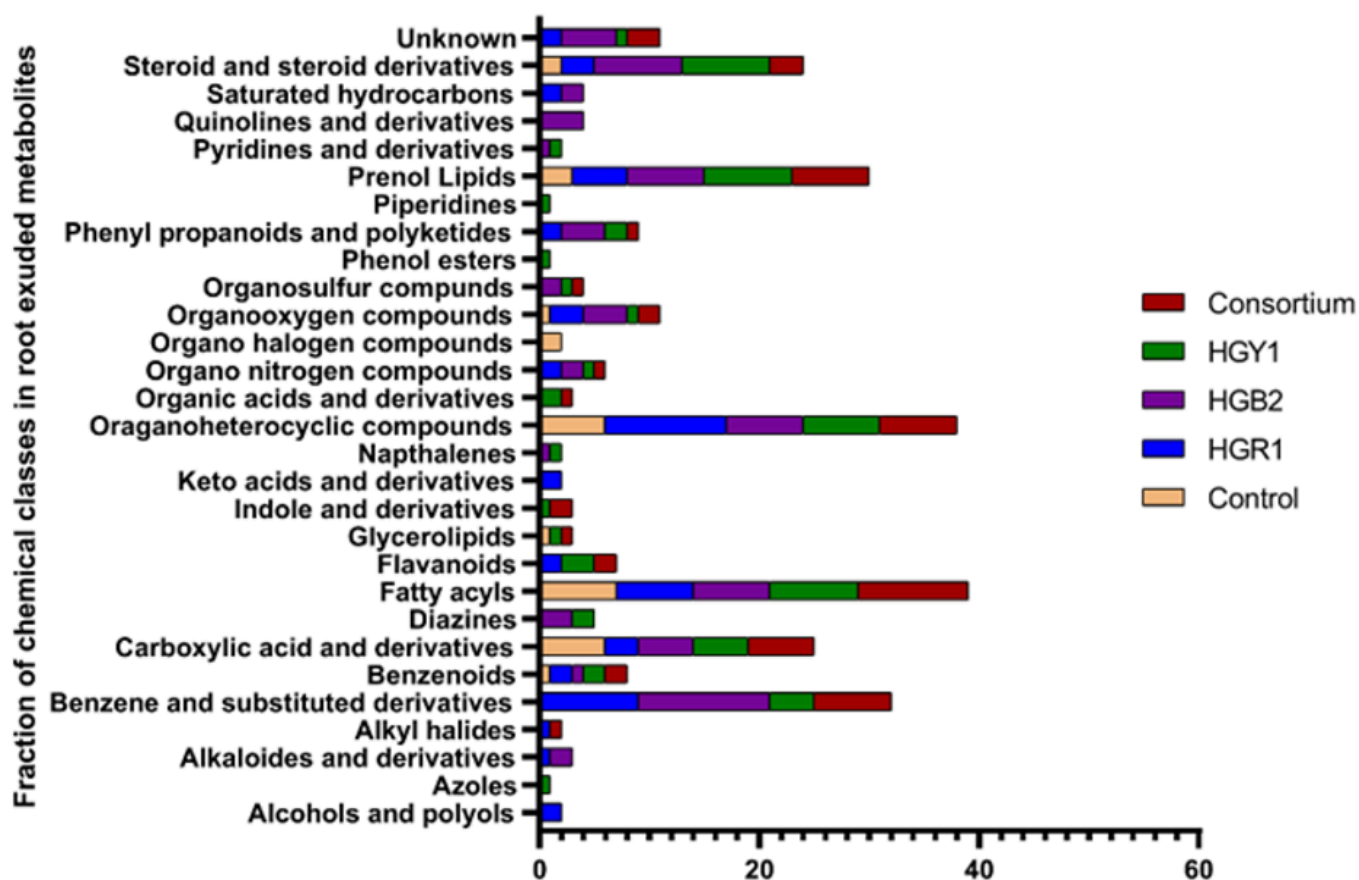


Figure 6

Root exudates profiling based on different classes of metabolites produced upon interaction under induced moisture deficit stress conditions HGR1 - *Rhizobium*sp, HGB2 - *P. indoloxydans*, HGY1 - *M. restricta*, Consortium (HGR1 + HGB2 + HGY1), and Control -uninoculated

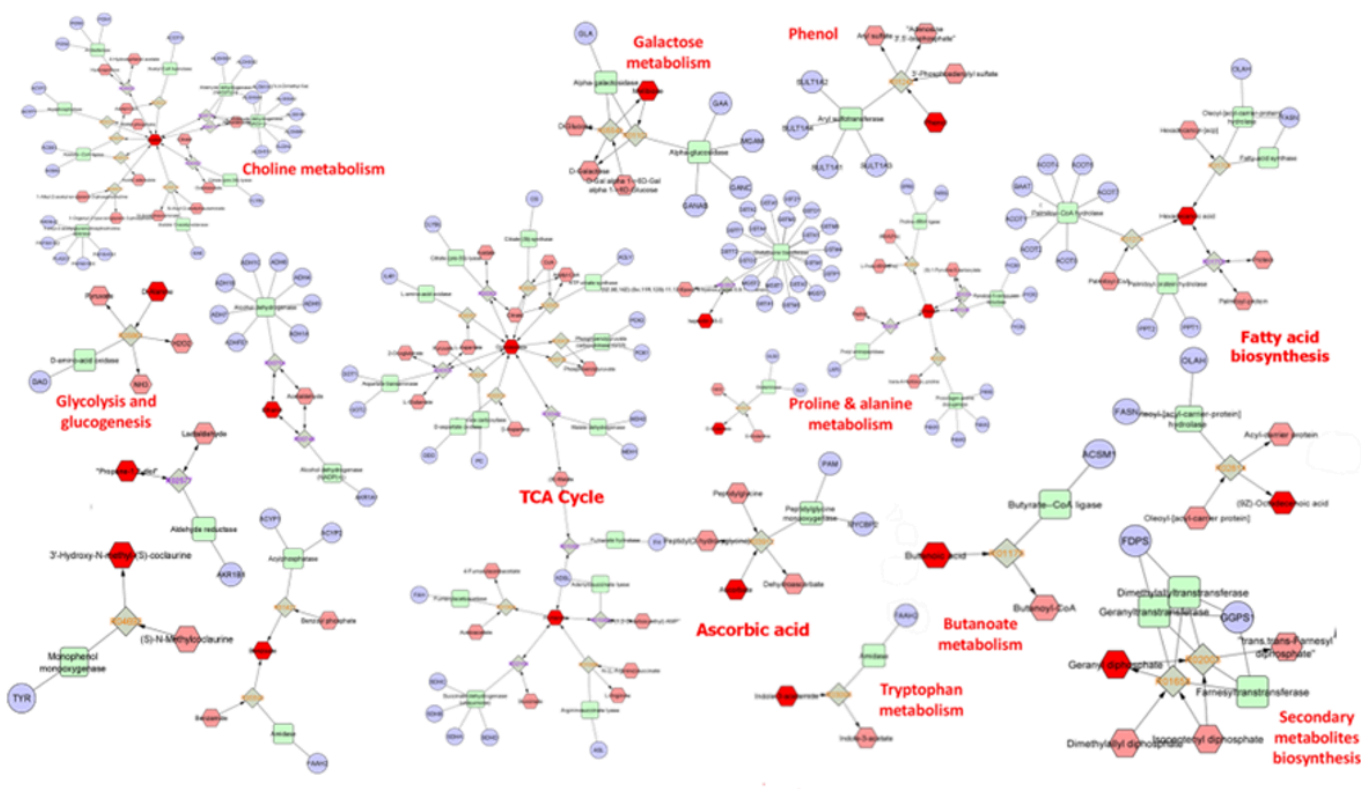


Figure 7

Presence of biochemical pathway expressed upon the interaction between potential NAPPs under induced moisture deficit stress conditions in Cytoscape 3.4.0. HGR1 - *Rhizobium*sp, HGB2 – *P. indoloxydans*, HGY1 – *M. restricta*

Supplementary Files

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