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ASSOCIATION OF ARBUSCULAR MYCORRHIZAL FUNGI, PLANT GROWTH PROMOTING FUNGI AND MYCORRHIZA HELPER BACTERIA TO ESTABLISH A TRIPARTITE INTERACTION AS A BOON FOR IMPROVEMENT OF PLANT GROWTH AND AUGMENTATION OF ALOIN AND ALOE EMODIN CONTENT IN *ALOE BARBADENSIS* MILL.

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ABSTRACT

The growing demand for sustainable agriculture has led to increased use of biofertilizers as eco-friendly alternatives to chemical fertilizers. Plant growth-promoting fungi (PGPF), arbuscular mycorrhizal fungi (AM), and mycorrhiza helper bacteria (MHB) are widely recognized for their ability to enhance plant growth and soil health. *Aloe barbadensis* Mill., a medicinal plant known for producing Aloin and Aloe emodin, holds significant pharmaceutical value. This study aimed to develop an effective microbial consortium-based biofertilizer and assess its impact on plant growth and secondary metabolite production in *A. barbadensis*. Based on plant growth-promoting traits, *Funneliformis mosseae* (AM), *Aspergillus terreus* (PGPF, MCC 1819), and *Bacillus tequilensis* (MHB, MCC 4174) were selected. Eight treatment sets were established to evaluate individual and combined effects. Results showed that the AM+MHB combination significantly improved soil nutrient status and primary plant growth. HPLC analysis revealed maximum Aloin content in AM+MHB-treated plants and highest Aloe emodin content in the AM+PGPF set. The AM+MHB combination demonstrated strong potential as a sustainable biofertilizer, enhancing both growth and metabolite yield in *Aloe barbadensis.* This microbial consortium offers an effective, eco-friendly strategy for improving medicinal plant cultivation and meeting global pharmaceutical demands.

Keywords: *Aloe barbadensis* Mill., Mycorrhizal Helper Bacteria

1. INTRODUCTION

The extensive use of agricultural chemicals by farmers to meet global food demands has led to a decline in beneficial microorganisms associated with crops and a significant reduction in soil microbial diversity, collectively known as the crop microbiome **[1]**. In response, the adoption of microbial biofertilizers is rapidly increasing **[1]**. Biofertilizers have emerged as a sustainable alternative to chemical fertilizers, offering a cost-effective solution due to their simple production process and significantly lower application expenses **[2]**. These biofertilizers comprise living soil microorganisms that actively enhance the availability and uptake of essential mineral nutrients by plants, thereby improving soil fertility and crop productivity **[3]**.

According to the World Health Organization (WHO), nearly 80% of the global population depends on traditional indigenous medicine for primary healthcare **[4]**. Medicinal plants have long served as a vital source of therapeutic compounds, used to treat a wide spectrum of diseases and ailments **[5-7]**. Presently, over 25% of pharmaceutical drugs prescribed in developed nations are derived from medicinal plants, driving an ever-growing global demand for these botanical resources **[7, 8]**.

Aloe barbadensis Mill. is among the most significant medicinal plants, valued for its therapeutic properties for decades **[9]**. It is abundant in secondary metabolites, making it widely utilized in pharmaceuticals, food products, and cosmetics due to its aromatic and medicinal qualities **[10]**. The gel extracted from *Aloe barbadensis* exhibits diverse bioactive properties, including anti-inflammatory, anticancer, antifungal, and antioxidant activities, along with antidiabetic, anti-ulcer, hypoglycemic, and immunomodulatory effects **[11]**.

This plant is a rich reservoir of phytochemicals, primarily anthraquinones, anthrones, and phenolic compounds, which contribute to its extensive pharmacological benefits **[12]**. Among these, anthraquinones are the most prominent secondary metabolites, with *Aloe barbadensis* Mill. containing approximately 32 anthraquinones and their derivatives. Aloin and Aloe emodin are the most abundant and biologically active constituents, making them key contributors to the plant's medicinal value **[13]**.

To boost the production of the highly medicinally valuable compounds Aloin and Aloe emodin in *Aloe barbadensis* Mill., we utilized various microbial combinations, including Arbuscular Mycorrhizal (AM) fungi, Mycorrhizal Helper Bacteria (MHB), and Plant Growth-Promoting Fungi (PGPF). AM fungi have been shown to significantly

enhance plant productivity by increasing the accumulation of secondary metabolites, such as polyphenols, alkaloids, flavonoids, vitamins, and lignans, all of which provide substantial health benefits **[7, 14]**. Phosphate-solubilizing rhizobacteria, recognized as MHB, are integral in improving phosphorus uptake in plants **[15]**. Research indicates that bacteria derived from mycorrhizal fungi can promote mycorrhizal infection, spore production, and plant pathogen resistance **[16]**. Additionally, some MHB, such as *Pseudomonas* species, function as Plant Growth-Promoting Rhizobacteria (PGPR), fostering plant growth through both direct and indirect mechanisms **[16]**. The relationship between PGPF and plant roots has been shown to improve growth, morphology, resource allocation, and mineral uptake, while simultaneously boosting reproductive fitness and enhancing crop yield **[17]**.

The aim of this study was to stimulate primary growth and increase the Aloin and Aloe emodin content in the leaves of *Aloe barbadensis* Mill. by applying suitable combination of AM fungi, MHB, and PGPF. This strategy seeks to develop a potent biofertilizer to promote sustainable agricultural practices. Considering this, the objective of the present study was to augment the primary growth of the plants and also to increase the amount of Aloin and Aloe emodin content per leaves of the plant by applying different microbial strains like AM, MHB and PGPF in different combinations so that they can be develop as an effective biofertilizer for more sustainable agricultural practices.

2. MATERIAL METHODS

2.1. Isolation and Characterization of AM fungi, PGPF and Mycorrhiza Helper Bacteria

Rhizospheric soil of the plants *Aloe barbadensis* Mill. were collected from the departmental garden of Botany, The University of Burdwan (23.2324° N, 87.8615° E) in the year 2023. Isolation of plant growth promoting fungi were done by using the protocol of Dey *et al*. **[18]**. Based on the Plant Growth Promoting (PGP) traits, we selected one best promising fungal strain and named as FSD2. Similarly, we isolated and characterized the arbuscular mycorrhizal fungal strains collected from the rhizospheric soil of the plant following the protocol of Dey *et al.* **[19]**. After performing characterization tests, we selected *Funneliformis mosseae* as the most promising strain. Isolation of mycorrhiza helper bacteria were done from the outer wall of AM fungal spores of *Funneliformis mosseae* (the most dominant AM fungal species we found on the rhizospheric soil of the plant *Aloe barbadensis* Mill.) following the protocol of Dey *et al.* **[19]**. After that, the bacterial strains were characterized on the basis of plant growth promoting traits and

finally, one strain was selected and named as ABM1.

2.2. Molecular Identification and construction of phylogenetic trees

The bacterial strain (ABM1) and the fungal strain (FSD2) were identified using 16S rDNA sequencing method from NCCS, Pune, India, after deposition. For better identification and their systematic position, the phylogenetic trees of the strains were constructed based on neighbor-joining method **[20]** using MEGAX software **[21]**.

After collecting the AM fungal spores from the rhizospheric soil, the AM fungal species were identified by using the 'Manual for Identification of Vesicular Arbuscular Mycorrhizal Fungi by Schenck **[22]**.

2.3.Application of AM fungi, MHB and PGPF on the plant

A pot experiment was conducted consisting of eight distinct experimental sets, incorporating two fungal strains (*Funneliformis mosseae* as AM fungi and *Aspergillus terreus* as PGPF) and one bacterial strain (*Bacillus tequilensis* as MHB) in various combinations. Prior to inoculating the soil with the different microbial strains, the soil in each treated pot was surface sterilized following the protocol outlined by Querejeta **[23]** to eliminate any pre-existing microorganisms. Among the eight sets, one was designated as the control set, with no microbial cultures applied. Three sets were prepared by inoculating the plants with a single strain of either the bacteria or fungi, named AM, MHB, and PGPF, respectively. Three additional sets involved inoculating the plants with two microbial strains in different combinations, labeled AM+MHB, AM+PGPF, and MHB+PGPF. The final set involved inoculating the plants with all three microbial strains, labeled AM+MHB+PGPF. The experimental pots were maintained for a duration of two years (2023-2025) in an artificial glasshouse chamber located in our departmental garden, where room temperatures (27–33°C) were carefully regulated. The plants were watered every two days with double-distilled water (CAS No. 7732-18-5, Sigma-Aldrich, USA). After two years of treatment (2023-2025), both the morphological and biochemical parameters of the plants were assessed. Five replicates were included for each treatment.

2.4.Phosphorous and Nitrogen content in soil and plant leaves

Phosphorus and nitrogen content in the soil and plant leaves were done by using the standard protocol of Khan *et al.* **[24]**.

2.5.Effect on morphological parameters of plant

The morphological parameters like leaf length (cm), root length (cm), fresh leaf weight (g), dry leaf weight (g), fresh gel

weight (g) etc. were recorded for both control as well as treated set.

2.6.Effect on biochemical parameters of plant

The estimation of chlorophyll content in the plants was carried out using the standard protocol outlined by Khan *et al.* **[24]**. The total carbohydrate and total protein content were determined following the protocol of Khan *et al.* **[24]**.

2.7.Estimation of Reactive oxygen species (ROS) and ROS scavenging enzymes

Reactive oxygen species, including the superoxide anion, were quantified using the method outlined by Wu *et al.* **[25]**, while hydrogen peroxide levels were measured following the protocol established by Velikova *et al.* **[26]**. The activities of defense enzymes, such as catalase, peroxidase, and superoxide dismutase, were determined using the standard protocol of Joshi *et al.* **[27]**.

2.8. Estimation of DPPH activity

The total antioxidant capacity (TAC) was evaluated by the ability of plant extracts to react to 2.2- diphenyl-1-picrylhydrazyl (DDPH) and quench free radicals **[28]**. The antioxidant activity percentage was calculated by using the formula of **[28]**.

Antioxidant activity (%) = [(AC-AE)/AC] x100

Where, AC is the absorbance of DPPH solution without extract (Methanol + DPPH) and AE is the absorbance of tested extract (Methanolic extract + DPPH).

2.9.Estimation of total phenol and anthraquinone content

For estimation of total phenol and anthraquinone content, we followed the protocol of Karamian and Asadbegy **[29]** and Sakulpanich *et al.* **[30]** respectively.

2.10. Quantification of Aloin and Aloe emodin by HPLC analysis followed by ESI-MS study

Leaf samples were prepared according to the standard protocol outlined by Okamura *et al.* **[31]**. Chromatographic analysis of the processed samples was conducted using an RP-HPLC system (Hitachi Chromaster equipped with a 5420 UV-Vis detector, Hitachi Corporation, Japan) and a C18 Cosmosil Reverse Phase column (250 mm × 4.6 mm). HPLC-grade methanol (Sigma-Aldrich, USA) and HPLC-grade water (Merck, USA) were used as the mobile phase in isocratic mode, with a 70:30 ratio and a flow rate of 0.7 mL/min for 10 minutes. The concentrations of Aloin and Aloe emodin in the samples were determined by comparing the retention times of the sample peaks with those of standard Aloin and Aloe emodin (Sigma-Aldrich, USA) at a detection wavelength of 293 nm. During HPLC analysis, fractions were collected from all treated sets, and ESI-MS analysis was performed to confirm the presence of Aloin and Aloe emodin in all treated sets.

2.11. Statistical Analysis

The results for each experimental sets were noted on the basis of regular observation using five replicates. The data were statistically assessed based on one-way ANOVA by Microsoft excel X. Different alphabets above each value signified significant differences at $P< 0.05$ by F-test.

3. RESULTS AND DISCUSSION

The use of herbal medicines for addressing various health challenges is rapidly expanding worldwide, with a tremendous surge in acceptance and public interest in natural therapies across both developing and developed countries **[32]**. Numerous plant species are utilized by various herbal industries nationwide, with *Aloe barbadensis* Mill. being one of the most important medicinal plants, revered for its therapeutic properties for decades **[33]**. The primary phytochemicals found in this plant are anthraquinones **[9]**. Approximately 32 anthraquinones and their derivatives have been identified in this plant, with Aloin and Aloe emodin being the most prominent active compounds **[13]**.

Prolonged use of chemical fertilizers can lead to significant soil acidification, nutrient imbalances, and the degradation of beneficial microorganisms in the rhizosphere **[34]**. In light of these concerns, we aim to enhance the production of the highly medicinally significant compounds, Aloin and Aloe emodin content in the leaves by applying various microbial strains such as Vesicular Arbuscular Mycorrhizal (AM) fungi, Mycorrhizal Helper Bacteria (MHB), and Plant Growth Promoting Fungi (PGPF) in different combinations. Our goal is to identify the most effective microbial combination that maximizes the production of these highly medicinally significant compounds in the near future.

3.1. Isolation and Characterization of AM fungi, PGPF and Mycorrhiza Helper Bacteria

Among AM fungi, the dominant species was *Funneliformis mosseae* (172±8 spores per 10 g of rhizospheric soil of the plants) which was followed by *Rhizophagus irregularis* (76±9 spores per 10 g of rhizospheric soil of the plants). Out of two bacterial strains identified from the outer wall of AM fungal spore *Funneliformis mosseae,* we selected the bacterial strain ABB1 for further studies.

After checking plant growth promoting traits, we selected the fungal strain FSD2 as the most potent strain.

3.2. Molecular Identification and construction of phylogenetic trees

The microbial (ABB1 and FSD2) strains were sent to NCMR-NCCS, Pune for molecular identification based on 16S rDNA and 18S rDNA sequencing respectively. They identified the isolate ABM1 as *Bacillus tequilensis* KCTC 13622(T) and FSD2 as *Aspergillus terreus*. The phylogenetic trees of the bacterial strain

ABB1 and fungal strain FSD2 were done in Mega X software **(Figure 1 and 2)**.

3.3. Phosphorous and Nitrogen content in soil and plant leaves

The available phosphorus and nitrogen content in the soil were significantly higher in all treated plants compared to the uninoculated control plants. The highest levels of available phosphorus and nitrogen were observed in the AM+MHB-treated plants, followed by the AM+MHB+PGPF-treated set **(Table 1)**. Similarly, the phosphorus and nitrogen content in the leaves of all treated sets showed a significant increase compared to the untreated control set. After completing the experiment, it was found that the leaves of the AM+MHB-treated set contained the highest amounts of phosphorus and nitrogen **(Table 1)**. It was previously reported that rhizospheric bacteria, including mycorrhizal helper bacteria, may improve plant nutrition by increasing the P and N content of the soil **[35]**. Gupta *et al.* **[36]** observed an increase in soil phosphorus availability following the application of Plant Growth Promoting Rhizobacteria in Aloe barbadensis Mill. According to Uzma *et al.* **[37]**, phosphate-solubilizing rhizobacteria release organic acids, enhancing phosphate availability and producing other growth-promoting substances, thereby stimulating overall plant growth.

Subsequently, we attempted to analyze the Phosphorus alongside Nitrogen concentration within foliage of the plants, and we observed that every microorganism-exposed grouping possessed maximal levels of phosphorus and nitrogen within their leaves compared to the untreated control set. Vafadar *et al.* **[38]** similarly demonstrated that combined inoculation of Growth-Promoting Rhizobacteria and Arbuscular Mycorrhizal fungi resulted in augmentation of nitrogen alongside phosphorus assimilation in *Punica granatum* saplings.

3.4. Morphological parameters

The experiment demonstrated that all microbial-inoculated *Aloe barbadensis* Mill. plants exhibited significant improvements in root length, leaf length, and both fresh and dry leaf gel weight compared to the control. The AM+MHB combination showed the greatest increases, followed by the tripartite plants. Among the single-strain treatments, PGPF outperformed both AM and MHB. In case of fresh and dry root weights, AM+MHB showed the highest gains, followed by AM+MHB+PGPF treated set **(Table 2)**. Previously, Gamalero *et al*. **[39]** demonstrated that the combined application of the MHB strain *Pseudomonas fluorescens* 92rk, the Plant Growth-Promoting Bacteria (PGPB) strain *Pseudomonas fluorescens* P190r, and *Glomus mosseae* BEG12 (92rk+P190r+BEG12) significantly

enhanced plant morphological growth. Similarly, Nacoon *et al.* **[40]** reported the beneficial effects of co-inoculation with an arbuscular mycorrhizal fungus (AMF) and phosphate-solubilizing bacteria (PSB) in improving the growth and productivity of sunchoke under field conditions.

3.5. Biochemical Parameters

All treated sets exhibited markedly elevated chlorophyll content compared to the control set **(Table 3)**. Notably, the highest levels of chlorophyll-a and chlorophyll-b were recorded in plants treated with the AM+MHB combination **(Table 3)**, followed closely by those subjected to the tripartite interaction (AM+MHB+PGPF). Among the unipartite treatments, the PGPF-treated plants demonstrated the greatest accumulation of both chlorophyll-a and chlorophyll-b, whereas the AM-treated set exhibited the lowest chlorophyll content **(Table 3)**. A similar pattern was observed in total chlorophyll content, with AM+MHB-treated plants exhibiting the maximum concentration **(Table 3)**. Within the single-agent treatments, the PGPF-treated set maintained the highest total chlorophyll level after 24 months of application **(Table 3)**. Vafadar *et al.* **[38]** suggested that increased chlorophyll levels in dual symbiotic plants likely enhance photosynthetic rates and biomass production. Similarly, Aseri *et al.* **[41]** reported the highest chlorophyll content in *Punica granatum* when co-inoculated with *Glomus mosseae* and *Azotobacter brasilense*.

In terms of total carbohydrate content, all inoculated sets displayed significantly enhanced carbohydrate levels relative to the control. The AM+PGPF treatment yielded the highest carbohydrate concentration **(Table 3)**. Among individual treatments, the PGPF-treated plants recorded the highest carbohydrate accumulation, followed by the MHB- and AM-treated sets, respectively **(Table 3)**. Regarding total protein content, all inoculated groups demonstrated a substantial increase in protein levels compared to the control group. The AM+MHB treatment resulted in the highest protein content, followed by the tripartite combination AM+MHB+PGPF **(Table 3)**. Among the unipartite treatments, the PGPF-treated plants exhibited the highest protein concentration, while the AM-treated set recorded the lowest protein level **(Table 3)**. This aligns with Khan *et al.* **[24]**, who found that elevated sugar content supports photosynthesis and enhances plant growth. Additionally, protein content was higher in all treated plants than in the control. Kousar *et al.* **[42]** reported increased protein levels in plants inoculated with *Pseudomonas aeruginosa*. Khan *et al.* **[24]** also noted that under stress, rhizospheric bacteria enhance

amino acid and protein levels, aiding ROS scavenging and maintaining photosynthetic efficiency.

3.6. Reactive Oxygen Species (ROS), ROS scavenging enzymes and Antioxidant capacity

After 24 months of inoculation, the levels of reactive oxygen species (ROS), specifically superoxide anions and hydrogen peroxide, were quantified from dried leaf extracts. The findings revealed that the control group exhibited the highest accumulation of both superoxide anions and hydrogen peroxide, followed by the AM-treated group. In contrast, the AM+MHB-treated plants recorded the lowest ROS levels, indicating a significant reduction in oxidative stress **(Table 4)**. Batool *et al.* **[43]** reported that PGPR application reduces ROS accumulation under stress, preventing oxidative damage. Ahmad *et al.* **[44]** highlighted that rhizospheric microbes mitigate ROS-induced degradation of proteins, nucleic acids, and lipids.

All three ROS-scavenging enzymes—Catalase, Peroxidase, and Superoxide Dismutase—showed a marked increase in activity across all inoculated sets compared to the uninoculated control. The AM+MHB treatment led to the most pronounced enhancement in the activity of all three enzymes, followed by the tripartite treatment (AM+MHB+PGPF) **(Table 4)**. Among the single-agent treatments, the PGPF-treated plants demonstrated the highest activity of Peroxidase, Catalase, and Superoxide Dismutase, whereas the AM-treated group exhibited the lowest enzymatic activity **(Table 4)**. ROS scavenging enzymes, including superoxide dismutase (SOD), catalase, and peroxidase, were significantly higher in all treated plants than in the control, supporting findings by Bano *et al.* **[45]**. The highest antioxidant activity was observed in AM+MHB-treated plants, likely due to increased sugar accumulation and enhanced antioxidant defense, ensuring cellular stability under stress **[42]**.

The antioxidant capacity of the plant leaf extracts was evaluated using the DPPH radical scavenging assay. The AM+MHB-treated set exhibited the highest antioxidant activity, followed closely by the AM+MHB+PGPF-treated group **(Table 5)**.

3.7. Estimation of Total Phenol from leaf extracts

Anthraquinones are a class of naturally occurring phenolic compounds **[46]**; therefore, it was essential to estimate the total phenolic content from the leaf extracts. Upon completion of the analysis, it was observed that the plants treated with AM+MHB exhibited the highest total phenolic content, followed by those treated with the AM+MHB+PGPF combination. Among the unipartite treatments, the PGPF-

treated plants recorded the highest phenolic content **(Table 6)**.

3.8. Estimation of Anthraquinone from leaf extract

Following the experiment, it was determined that the AM+MHB-treated plants contained the highest anthraquinone content (8.98 mg g^{-1} DW), while the MHB+PGPF-treated set exhibited the lowest level (4.98 mg g^{-1} DW) among the bipartite interactions. Among the unipartite treatments, the highest anthraquinone content was observed in the PGPF-treated plants **(Table 6)**.

3.9. HPLC analysis of our targeted anthraquinone (Aloin and Aloe emodin) from the extracts

HPLC analysis was conducted to quantify the targeted anthraquinones—Aloin and Aloe emodin—from the plant samples. The results revealed that the concentrations of both Aloin and Aloe emodin increased in all microbially inoculated sets compared to the uninoculated control group. The highest Aloin content was observed in the AM+MHB-treated set, showing an increase of up to 261.45% compared to the untreated control **(Figure 3)**, followed by the AM+MHB+PGPF-treated set, which showed an increase of up to 228.12%. Among the unipartite treatments, the highest Aloin content was recorded in the PGPF-treated set, with an increase of up to 60.41% over the control, followed by the MHB-treated set; the lowest Aloin level was found in the AM-treated plants **(Table 6) (Figure 3)**.

Similarly, the Aloe emodin content in all microbially treated plants was significantly elevated compared to the uninoculated control. The AM+PGPF-treated plants produced the highest amount of Aloe emodin, showing an increase of up to 296.38% relative to the control, followed by the unipartite PGPF-treated set. Among the single-treatment groups, Aloe emodin content was highest in PGPF-treated plants, followed by those treated with MHB **(Table 6)**. Secondary metabolite analysis revealed a significant increase in phenolic and anthraquinone content in all treated plants, with the highest levels in AM+MHB, followed by AM+MHB+PGPF. Enhanced phosphorus uptake has been linked to increased phenolic pathways **[47]**, and microbial treatments have similarly boosted phenol levels **[48, 49]**. HPLC confirmed increased Aloin and Aloe emodin in all microbial treatments. AM+MHB yielded the highest Aloin content, supporting Gupta *et al.* **[36]** and Sharma *et al.* **[50]**, while AM+PGPF produced the most Aloe emodin, aligning with Sun *et al.* **[51]**. Aloe emodin levels increased as Aloin decreased, likely due to its *in vitro* oxidation **[52]**.

3.10. Study of ESI MS of the plant extracts after collecting the fraction during HPLC analysis

After collecting the fractions during HPLC analysis, the HPLC/ESI-MS (Electrospray Ionization Mass Spectrometry) study confirmed the presence of Aloin in all eight treatment sets. The detected molecular weights were as follows: 301.1610 in the control, 301.1504 in AM, 301.1504 in MHB, 301.1504 in PGPF, 301.1504 in AM+MHB, 301.1610 in AM+PGPF, 301.1539 in MHB+PGPF, and 301.1504 in AM+MHB+PGPF. These values closely correspond to the molecular weight of standard Aloin (301.1504), thereby confirming its identity across all samples.

Similarly, the HPLC/ESI-MS analysis confirmed the presence of Aloe emodin in all eight sets. The observed molecular weights were: 245.0748 in the control, 245.0812 in AM, 245.0780 in MHB, 245.0780 in PGPF, 245.0684 in AM+MHB, 245.0748 in AM+PGPF, 245.0748 in MHB+PGPF, and 245.0684 in AM+MHB+PGPF. These values closely align with the molecular weight of standard Aloe emodin (245.0940), validating its presence in each treatment group.

3.11. Corelation

We analyzed the correlation between soil nutrients (N and P) and plant morphology **(Figure 4)**. Fresh gel weight showed a strong positive correlation with soil P and N, aligning with Ahmad *et al.* **[53]**. AM fungi enhance P uptake via phosphatases and transporter proteins **[51]**. No significant correlation was found for fresh rind weight, but dry gel weight correlated with soil N, consistent with Chowdhury *et al.* **[54].**

A positive link was observed between soil nutrients and anthraquinone content (Figure 5). Aloin increased in all treated sets, supporting Sun *et al.* **[51]** and Rai *et al.* **[55]**, as P solubilization enhanced photosynthesis and carbon acquisition. Nitrogen also boosted Aloin levels, as reported by Saliqehdar *et al.* **[56]** and Chowdhury *et al.* **[57]**. However, Aloe emodin showed no correlation with soil P.

Total chlorophyll and carbohydrate content strongly correlated with soil nutrients, supporting Razaq *et al.* **[58]** and Yadav *et al.* **[59]**, who linked N and P to enhanced photosynthesis and growth. Total protein content correlated with soil P but not N **(Figure 5)**.

4. CONCLUSION

Selecting an optimal microbial consortium is crucial for enhancing anthraquinone production in *Aloe barbadensis* Mill. By applying AM, MHB, and PGPF combinations, we significantly boosted plant growth and Aloin and Aloe emodin content compared to the control. Microbial application also improved soil nutrient levels, aligning with Uzma *et al.* **[37]**, who

reported that phosphate-solubilizing rhizobacteria enhance P availability and promote plant growth. In our study, the AM+MHB combination yielded the highest morphological growth and soil nutrient enrichment. HPLC confirmed maximum Aloin accumulation in AM+MHB-treated plants. Given the global demand for *Aloe barbadensis* Mill. gel, the AM fungus (*Funneliformis mosseae*) and MHB (*Bacillus tequilensis*) consortium holds promise as a sustainable biofertilizer for enhanced cultivation. However, further in-depth studies are required to validate and establish this microbial consortium as a potent and sustainable biofertilizer for large-scale agricultural applications in near future.

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6. **AUTHOR CONTRIBUTIONS**

Shinjan Dey provided the main concept of the work. Literature study and lab work were done by Sikha Dutta. Shinjan Dey and Debapriya Choudhury helped in formatting. All authors read and approved the manuscript.

7. **CONFLICTS OF INTEREST**

The authors report no financial or any other conflicts of interest in this work.

8. **ETHICAL APPROVALS**

This study does not involve experiments on animals or human subjects.

9. **DATA AVAILABILITY**

All the data is available with the authors and shall be provided upon request.

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Table 1: Morphological parameters of the treated plants

TREATMENTS	Available Phosphorus (kg ha^{-1})	Available Nitrogen (kg ha^{-1})	Phosphorus uptake in leaves (mg $plant^{-1}$)	Nitrogen uptake in leaves (mg $plant^{-1}$)
CONTROL	52.63±1.1^{a}	220.87±0.7^{a}	5.76±0.3^{a}	11.8±0.4^{a}
AM	57.12±0.8^{b}	250.88±1.1^{b}	5.98±0.2^{b}	12.2±0.5^{b}
MHB	59.81±0.7^{b}	275.97±1.3^{c}	6.24±0.4^{b}	12.9±0.3^{b}
PGPF	61.15±1.4^{b}	301.06±1.8^{d}	7.97±0.7^{c}	14.7±0.7^{c}
AM+ MHB	75.00±1.7^{c}	376.32±2.7^{e}	12.6±0.9^{d}	18.9±0.3^{d}
AM +PGPF	67.87±1.2^{bc}	326.14±2.1^{de}	8.9±0.5^{c}	16.4±0.8^{cd}
MHB+PGPF	65.18±1.5^{bc}	319.86±1.8^{de}	8.2±0.8^{c}	12.6±0.7^{b}
AM+MHB+PGPF	71.31±1.6^{bc}	351.23±1.2^{de}	10.5±0.5^{cd}	15.7±0.4^{cd}

(The data represent the mean of five replicates) [Values are expressed as mean ± SD (n = 5); statistically significant at P < 0.05 when compared to the control group]. Means followed by the same letter do not differ significantly according to the Least Significant Difference (LSD) test at p<0.05.

Table 2: Morphological parameters of the treated plants

Treatments	Root Length (cm)	Leaf Length (cm)	Fresh Leaf weight (g)	Fresh gel weight (g)	Fresh rind weight (g)	Dry leaf weight (g)	Dry gel weight (g)	Dry rind weight (g)
CONTROL	12.2±0.3^{a}	24.5±0.3^{a}	169.76±0.57^{a}	71.87±0.54^{a}	98.87±0.12^{a}	2.43±0.09^{a}	0.76±0.01^{a}	1.67±0.07^{a}
AM	13.3±0.4^{b}	26.7±0.6^{b}	205.43±0.45^{b}	97.98±0.91^{b}	102.80±0.49^{a}	2.96±0.12^{b}	0.83±0.02^{a}	2.14±0.08^{b}
MHB	13.9±0.5^{b}	27.4±0.6^{b}	246.09±0.67^{c}	112.65±0.51^{c}	127.45±0.65^{b}	3.88±0.15^{c}	1.45±0.04^{b}	2.43±0.07^{c}
PGPF	15.7±0.5^{c}	28.2±0.5^{c}	298.76±0.31^{d}	136.76±0.76^{d}	145.87±0.48^{c}	4.35±0.21^{d}	1.75±0.04^{c}	2.61±0.09^{d}
AM+MHB	16.3±0.6^{d}	31.3±1.1^{d}	387.98±0.58^{e}	166.65±0.44^{e}	209.40±0.64^{d}	4.86±0.19^{e}	2.08±0.06^{d}	2.78±0.17^{e}
AM+PGPF	15.9±0.5^{cd}	29.1±0.3^{cd}	305.79±0.4^{de}	154.76±0.89^{de}	149.67±0.88^{c}	4.39±0.23^{d}	1.98±0.07^{cd}	2.41±0.14^{c}
MHB+PGPF	15.8±0.6^{cd}	29.3±0.^{5cd}	271.86±0.78^{cd}	130.87±0.37^{d}	139.84±0.98^{c}	4.36±0.19^{d}	1.87±0.03^{cd}	2.49±0.19^{c}
AM+MHB+PGPF	16.2±0.4^{d}	30.2±1.3^{cd}	356.56±0.44d^{de}	158.54±0.66^{de}	195.80±0.92^{d}	4.62±0.21^{de}	2.06±0.04^{d}	2.56±0.21^{d}

(The data represent the mean of five replicates) [Values are expressed as mean ± SD (n = 5); statistically significant at P < 0.05 when compared to the control group]. Means followed by the same letter do not differ significantly according to the Least Significant Difference (LSD) test at p<0.05.

Table 3: Biochemical parameters of the treated plants

Treatments	Chlorophyll-a (mg g^{-1} FW)	Chlorophyll-b (mg g^{-1} FW)	Total Chlorophyll (mg g^{-1} FW)	Total carbohydrate (mg g^{-1}DW)	Total Protein (mg g^{-1}DW)
CONTROL	14.22±0.34^{a}	3.1±0.05^{a}	17.32±0.65^{a}	21.76±0.19^{a}	19.761±0.08^{a}
AM	16.1±0.38^{b}	3.75±0.03^{b}	19.76±0.76^{b}	25.43±0.22^{b}	19.842±0.1^{a}
MHB	16.8±0.78^{b}	4.96±0.19^{c}	21.76±0.51^{c}	26.09±0.23^{b}	22.5±0.08^{b}
PGPF	19.82±0.56^{c}	6.65±0.09^{d}	26.42±0.84^{d}	29.46±0.25^{c}	23.296±0.06^{c}
AM+MHB	28.3±0.76^{d}	9.1±0.2^{e}	37.4±1.19^{e}	31.08±0.31^{d}	26.798±0.04^{d}
AM+PGPF	25.7±0.68^{cd}	7.84±0.12^{d}	33.54±0.84^{de}	31.79±0.27^{d}	24.976±0.09^{c}
MHB+PGPF	21.54±0.96^{c}	6.98±0.10^{d}	28.52±0.76^{d}	28.86±0.17^{c}	22.83±0.11^{b}
AM+MHB+PGPF	27.4±0.71^{d}	8.95±0.16^{e}	36.35±0.99^{e}	29.56±0.25^{c}	23.581±0.07^{c}

(The data represent the mean of five replicates) [Values are expressed as mean ± SD (n = 5); statistically significant at P < 0.05 when compared to the control group]. Means followed by the same letter do not differ significantly according to the Least Significant Difference (LSD) test at p<0.05.

Table 4: Estimation of ROS and ROS scavenging enzymes of the treated plants

Treatments	Superoxide Anion Content (µg g^{-1} DW)	Hydrogen Peroxide Content (µM g^{-1} DW)	Peroxidase (Ug^{-1} $DWsec^{-1}$)	Catalase (Ug^{-1} $DWsec^{-1}$)	Superoxide dismutase (Ug^{-1} $DWsec^{-1}$)
CONTROL	25.098 ± 0.005^{a}	10.085 ± 0.003^{a}	0.354 ± 0.009^{a}	0.054 ± 0.0007^{a}	0.317 ± 0.005^{a}
AM	25.112 ± 0.007^{a}	10.081 ± 0.001^{a}	0.366 ± 0.023^{a}	0.055 ± 0.0009^{a}	0.322 ± 0.008^{a}
MHB	24.064 ± 0.002^{a}	9.055 ± 0.004^{b}	0.501 ± 0.034^{b}	0.062 ± 0.0014^{b}	0.338 ± 0.018^{b}
PGPF	23.648 ± 0.003^{b}	8.683 ± 0.006^{c}	0.579 ± 0.054^{c}	0.072 ± 0.0006^{c}	0.384 ± 0.026^{c}
AM+MHB	20.836 ± 0.001^{c}	7.158 ± 0.005^{d}	0.689 ± 0.068^{d}	0.089 ± 0.0017^{d}	0.476 ± 0.033^{d}
AM+PGPF	21.02 ± 0.006^{c}	8.185 ± 0.003^{cd}	0.684 ± 0.048^{d}	0.085 ± 0.0034^{cd}	0.468 ± 0.056^{d}
MHB+PGPF	23.695 ± 0.005^{b}	9.042 ± 0.008^{b}	0.503 ± 0.039^{b}	0.068 ± 0.0021^{bc}	0.403 ± 0.037^{cd}
AM+MHB+PGPF	22.814 ± 0.011^{bc}	8.002 ± 0.006^{cd}	0.644 ± 0.027^{d}	0.078 ± 0.0029^{cd}	0.449 ± 0.046^{cd}

(The data represent the mean of five replicates) [Values are expressed as mean ± SD (n = 5); statistically significant at P < 0.05 when compared to the control group]. Means followed by the same letter do not differ significantly according to the Least Significant Difference (LSD) test at p<0.05.

Table 5: Total Antioxidant Capacity (TAC) of the plant extracts

Conc. of Methanolic plant extract (mg ml^{-1})	% Antioxidant activity (% Inhibition of DPPH radicals) Treated Sets							
	CONTROL	AM	MHB	PGPF	AM+MHB	AM+PGPF	MHB+PGPF	AM+MHB +PGPF
0.50	11.37^{a} ±0.43	12.76^{b} ±0.65	17.76^{c} ±0.87	22.7^{d} ±0.43	32.5^{e} ±0.56	29.87^{de} ±0.55	23.65^{d} ±0.32	30.87^{de} ±0.48
1.0	15.29^{a} ±0.54	30.19^{b} ±0.22	40.58^{c} ±0.44	55.68^{d} ±0.65	67.64^{e} ±0.43	61.37^{de} ±0.34	58.62^{de} ±0.77	60.98^{de} ±0.79

Table 6: Estimation of total phenol, anthraquinone, Aloin and Aloe emodin of the treated plants

Treatments	Total Phenol (mg GAE g^{-1} DW)	Total Anthraquinone (mg g^{-1} DW)	Aloin (mg g^{-1} DW)	Aloe emodin (mg g^{-1} DW)
CONTROL	42.67±1.6^{a}	2.55±0.1^{a}	0.96±0.03^{a}	0.83±0.12^{a}
AM	48.98±1.2^{b}	3.64±0.06^{b}	0.98±0.06^{a}	0.96±0.08^{b}
MHB	64.76±1.8^{c}	3.97±0.09^{c}	0.99±0.07^{a}	1.54±0.23^{c}
PGPF	78.98±1.9^{d}	6.69±0.16^{d}	1.54±0.18^{b}	3.17±0.06^{d}
AM+MHB	110.54±2.1^{e}	8.98±0.27^{e}	3.47±0.18^{c}	2.46±0.19^{cd}
AM+PGPF	97.76±1.7^{de}	6.97±0.14^{de}	2.14±0.05^{bc}	3.29±0.16^{d}
MHB+PGPF	96.45±1.9^{de}	4.98±0.21^{cd}	1.67±0.02^{b}	1.27±0.12^{c}
AM+MHB+PGPF	103.65±1.9^{de}	7.71±0.29^{de}	3.15±0.23^{c}	2.18±0.19^{cd}

(The data represent the mean of five replicates) [Values are expressed as mean ± SD (n = 5); statistically significant at P < 0.05 when compared to the control group]. Means followed by the same letter do not differ significantly according to the Least Significant Difference (LSD) test at p<0.05.

FIGURES

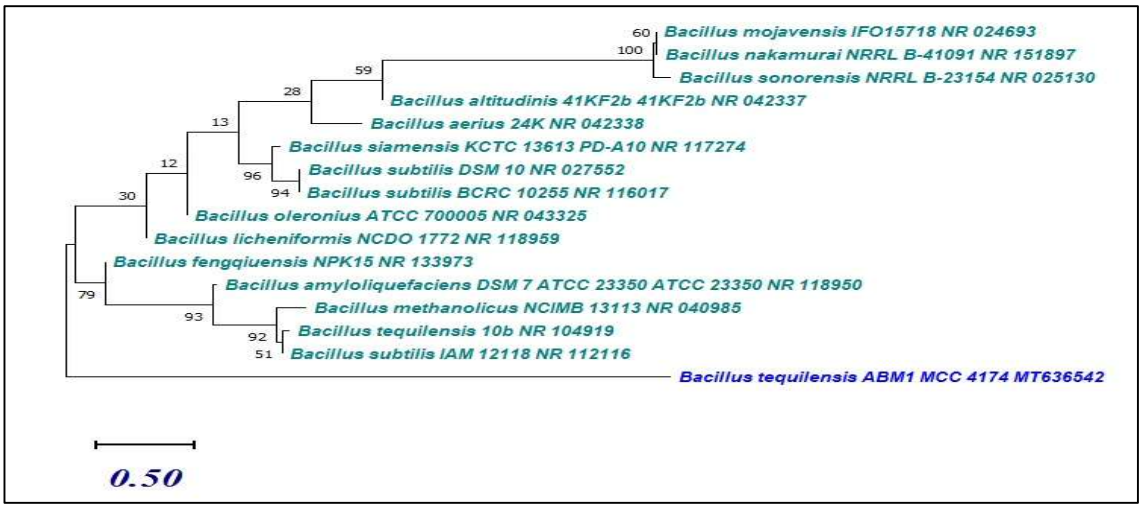

Figure 1: Phylogenetic tree of the bacterial strain ABM1

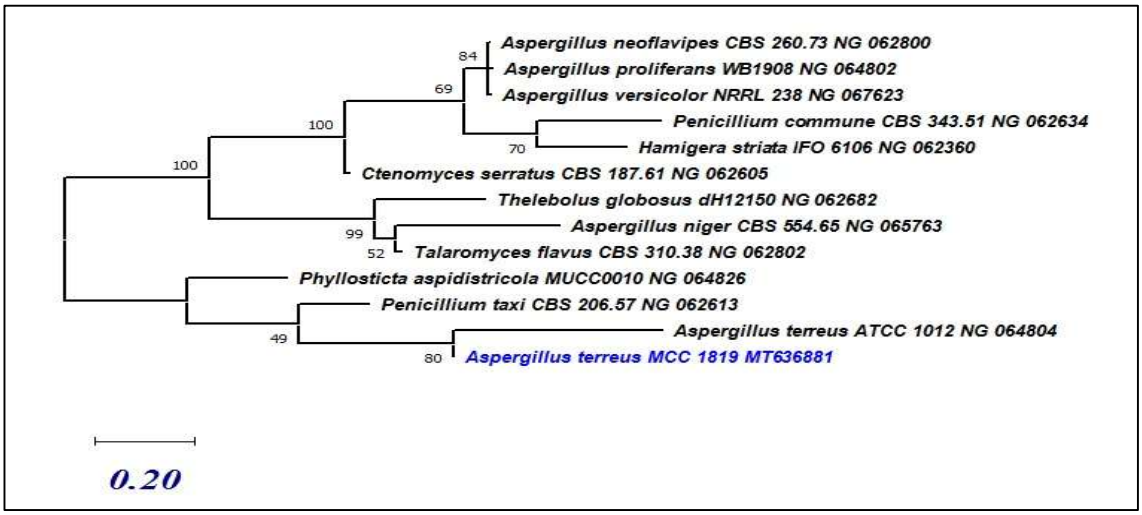

Figure 2: Phylogenetic tree of the fungal strain FSD2

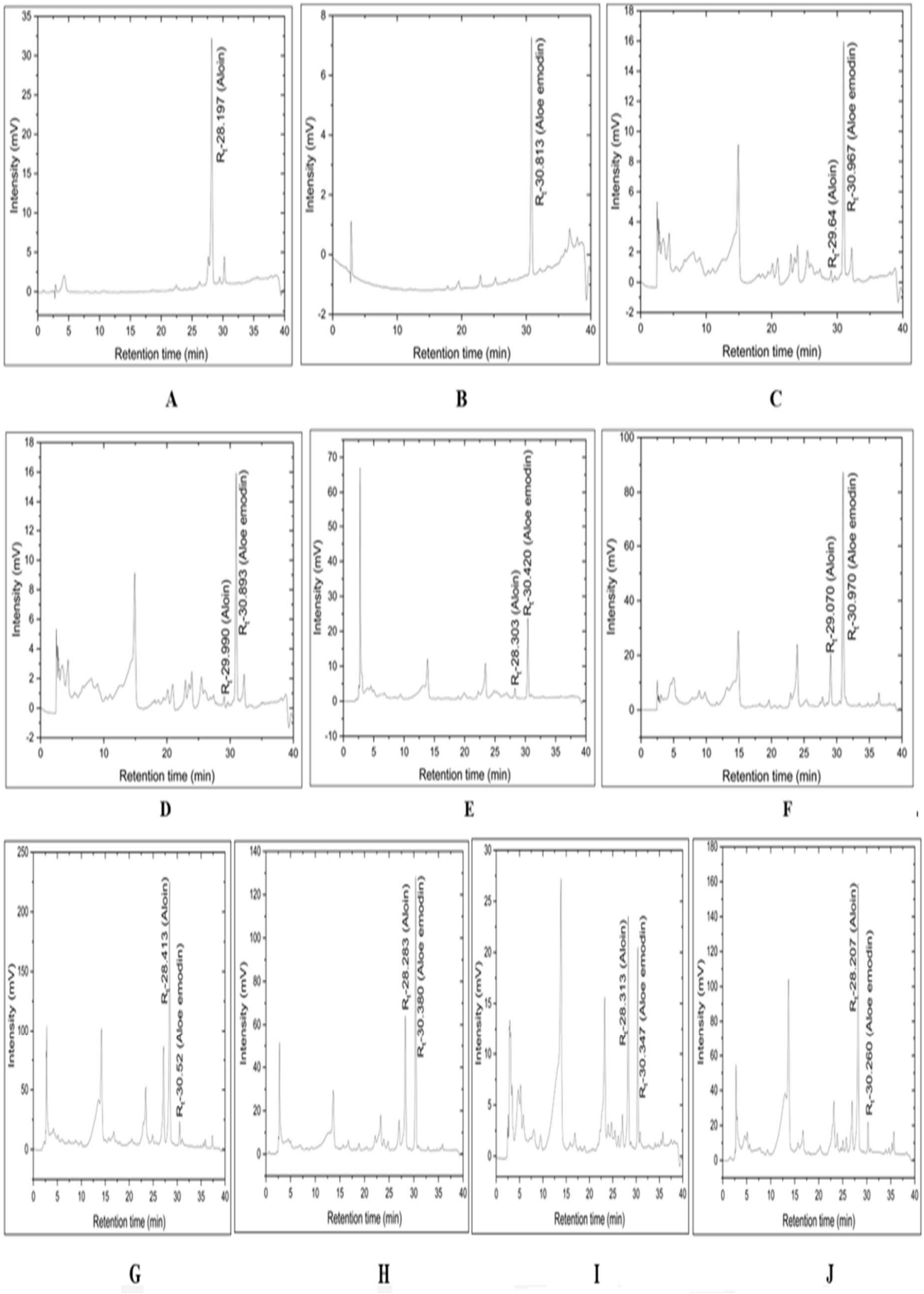

Figure 3: HPLC chromatograms of different set of plants. A- Standard Aloin; B-Standard Aloe emodin; C- Control; D-AM; E-MHB; F-PGPF; G- AM+MHB; H-AM+ PGPF; I- MHB+PGPF; J- AM+MHB+PGPF

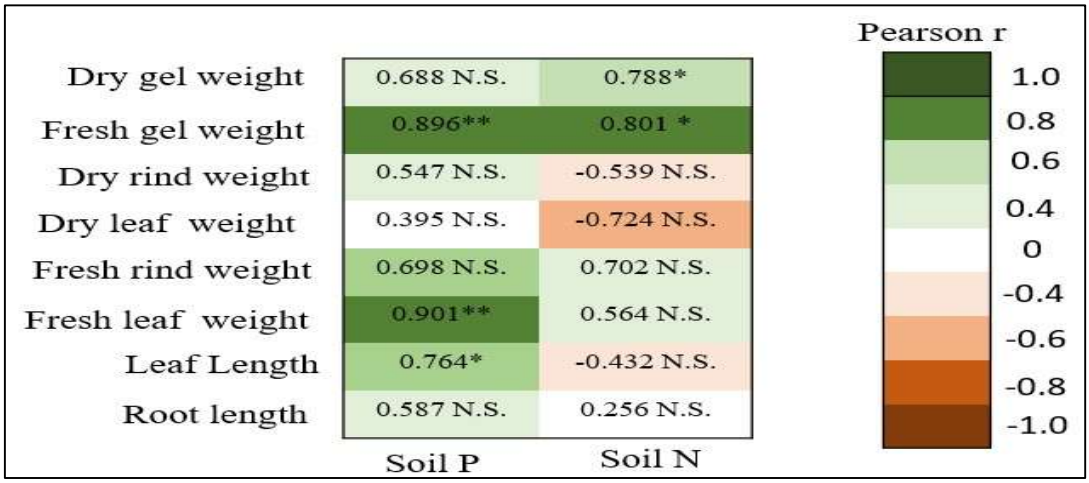

Figure 4: A correlogram showing Pearson's correlation coefficients between soil phosphorus and nitrogen and various plant morphological traits. Asterisks denote the levels of statistical significance: * p < 0.05, ** p < 0.01

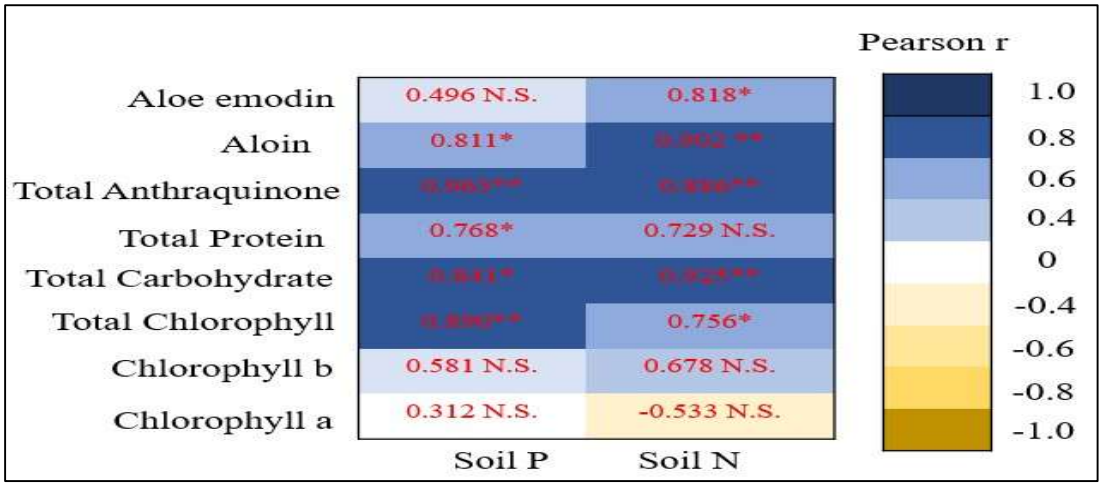

Figure 5: A correlogram showing Pearson's correlation coefficients between soil phosphorus, nitrogen, and a range of plant biochemical traits and secondary metabolites. Asterisks indicate the significance levels of the correlations: * p < 0.05, ** p < 0.01