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EFFECT OF MICROBIAL BIOINOCULATION ON THE BIOACTIVE SECONDARY METABOLITES PRODUCTION OF *ELAEAGNUS LATIFOLIA* L. SEEDLINGS

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Keywords:

Arbuscular mycorrhizal fungi, Bio-inoculation, *Elaeagnus latifolia*, Phenol, Flavonoid and DPPH assay

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ABSTRACT: The role of rhizospheric symbiotic Arbuscular Mycorrhizal (AM) fungus, non-AM fungus and bacteria in biochemical metabolites production of *E. latifolia* L. seedlings was analysed. The seedlings were inoculated with AM fungal strain (*Glomus* species), non-AM fungus (*Trichoderma harzianum*, Th-13) and bacteria (*Pseudomonas* species, Ps-1) in combined form (both, dual and triple/tripartite consortium) and analysed for their effect on the total bioactive chemical metabolites production of the target plant species. Qualitative estimation of the secondary metabolites in the said species was done and the following compounds were present *viz.* alkaloids, tannins, flavonoids, lignin *etc*. Quantitative estimation of phenolics, flavonoids, anti radical scavenging activity were observed using DPPH assay. The plant carbon and Phosphorus content were also analysed. The maximum phenolic content was obtained in the ethanolic extracts of T3 (173±0.025 mg/g) treated seedlings and the maximum flavonoid content was obtained in the ethanolic extracts of T8 (85.42±0.015 mg/g). Analyses of the free radical scavenging activity revealed that, the highest % inhibition activity was showed by T4 treatment (95.58±0.02) and the least was found in T5 treatment (76.17±0.03). Similar trend with diminutive variation in all studied parameters as cited above was also observed in other alone and combined treatments than control seedlings which are discussed in detail in this paper.

INTRODUCTION: The standard method for examining plant growth and production is thought to be plant growth analysis [1]. Yield and growth are products of numerous metabolic processes that are influenced by hereditary and environmental variables. Understanding growth patterns helps us understand how plants collect dry matter, as well as the factors that influence a plant's ability to produce more or less in a population or on an individual basis [2].

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Numerous elements, including the environment, edaphic conditions, meteorological conditions, altitudinal differences, and most importantly, the rhizospheric bacteria associated with the plant species, influence plant growth. Microorganisms play a fundamental role in the cycling of inorganic and organic P in the rhizosphere [3].

Plant secondary metabolites play a variety of functions such as in plant growth and developmental processes, innate immunity[4], defense response signalling [5], and response to environmental stresses [6]. In addition, PSMs also have important functions such as repelling pests and pathogens, acting as signals for symbiosis between plants and microbes, and modifying microbial communities associated with hosts [7, 8].

Arbuscular mycorrhizal (AM) symbiosis is known to bring a wide range of benefits to the host plant in both agricultural production systems and natural ecosystems. The main effect of AM fungi is their capacity to supply mineral nutrients that are relatively immobile in the soil, particularly P and trace elements; they achieve this by exuding organic anions and phosphatase enzymes, and by modifying the pH around their hyphae [9].

Many plant growth promoting rhizobacteria (PGPR) are also known to stimulate plant growth through direct or indirect interactions with plant roots. PGPR, primarily pseudomonades, are known to aid in the release of phosphate ions from both organic and sparingly soluble inorganic sources of P (by the secretion of phosphatase enzymes) in soil with low P availability. Both fungi and bacteria are involved in biocontrol activities, with the fungus [10].

Trichoderma having a substantial influence on the management of plant diseases. Because it may form a mycorriza-like relationship with plants, trichoderma is commonly employed as a biocontrol agent against phytopathogenic fungi as well as a biofertilizer [11]. Thus, it can be speculated that a synergistic interaction of all these microorganisms in the rhizosphere of plant species has a direct impact on the overall growth and development of the plant species.

The majority of plant species have symbiotic relationships with arbuscular mycorrhizal fungus (AMF). These fungi improve soil quality and structure by enhancing the uptake of relatively immobile minerals, especially phosphorus and other micronutrients. Numerous additional soil species are in contact with mycorrhizal fungi in the bulk soil, rhizosphere, and roots. These interactions could be mutualistic or stimulatory; some could be obviously competitive. By increasing the amount of nutrients that can be absorbed, these bio-elicitors improve the creation of primary products and provide more resources for the synthesis of secondary metabolites [12, 13].

Plants and microbial endophytes have a symbiotic connection in which the endophytes live inside the host plant and do not clearly show any signs of illness. Plants of all shapes and sizes, including flowers, seeds, leaves, roots, and stems, have been found to harbor bacterial endophytes. They create both primary and secondary metabolites, which have a variety of biological functions and can significantly affect the metabolism of the host plant.

Bacterial endophytes, for instance, have the ability to improve host plant health, increase growth and yield in a variety of plant species, and increase resistance to different diseases and environmental stressors [14].

Symbiotic and non-symbiotic plant growth promoters (PGPRs) that work by producing plant hormones such auxins, cytokinins, gibberellins, ethylene, and abscisic acid are examples of direct plant growth promotion. Production of auxins, such as indole-3-ethanol or indole-3-acetic acid (IAA), has been documented for a number of bacterial taxa.

Additionally, PGPR strengthens resistance to stress, stabilizes soil aggregates, improves soil structure and organic matter content, and solubilizes mineral phosphates and other nutrients. PGPR increases the amount of soil organic N and other nutrients that are retained in the plant-soil system, hence decreasing the requirement for fertilizer N and P and improving nutrient release [15].

MATERIALS AND METHODS:

Collection of Plant, Soil and Root Samples: Survey was made at selected areas for the collection of plant specimen, *Elaeagnus latifolia* L., **Fig. 1** which is known for its symbiotic relationship with several beneficial microbiotas.

The rhizospheric soil samples of the plant are also collected to investigate and enumerate important microbial associations. The collected plant specimens were preserved in herbarium sheet for further identification. Rhizospheric soil samples (at least three samples at each location) were taken by digging out a small amount of soil (500 g) adjacent to plant roots up to the depth of 15-30 cm.

The collected soil samples were kept in pre-sterilized polythene at 4 °C to estimate the physico-chemical parameters of soil, mycorrhizal colonization and quantification of VAM fungi as well as actinorhizal and endophytic associations.

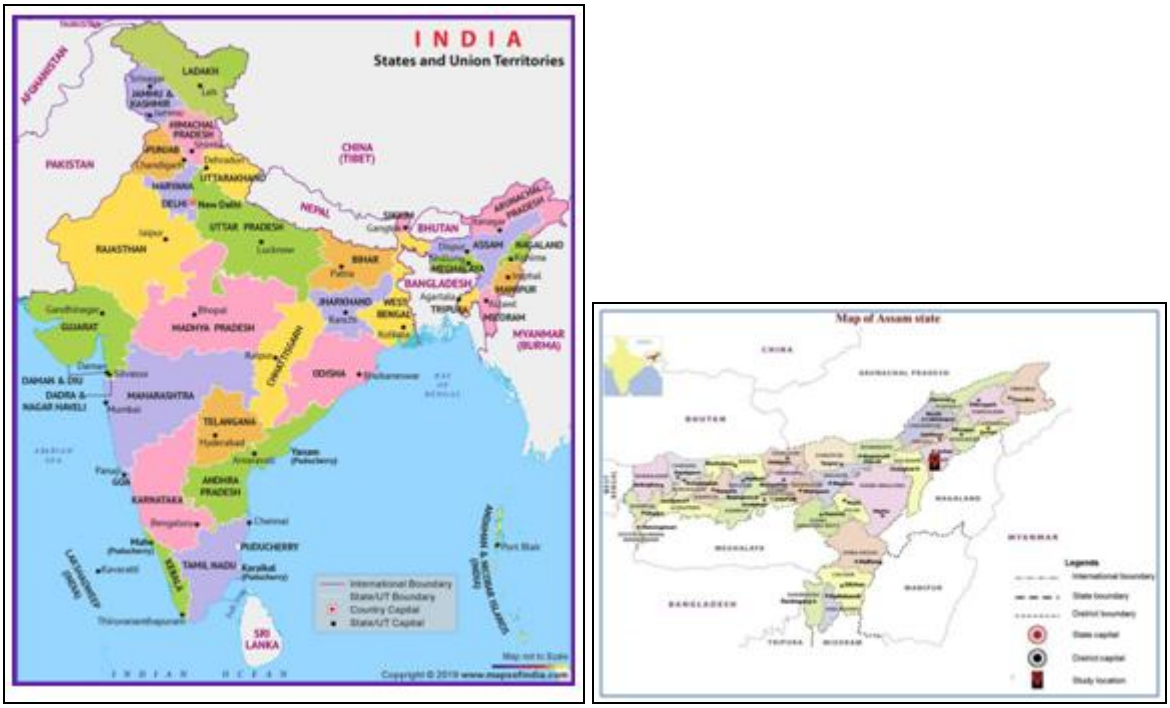

FIG. 1: STUDY SITE

Isolation and Characterization of Rhizospheric Microorganisms: In the present investigation, a culture-based approach was primarily used to isolate and characterize the rhizospheric microbes of selected study points. For this, randomly collected soil samples were mixed properly and passed through a 2.0 mm sieve to remove the debris. 1.0 g of soil was then suspended in 10 ml of SDW and incubated in an orbital shaking incubator at 28 °C with periodic shaking at 200 rpm for 30 minutes. 10-fold series dilutions were prepared serially by taking 1 ml of the soil suspension and dispensing it into 9 ml of SDW. Soil particles were allowed to settle and serial dilutions were prepared using SDW. The soil suspension of required dilution was then inoculated into culture media and incubated at optimum temperature for maximum growth of rhizosphere microbes. The colony forming units (cfu) were counted after proper incubation period was over. Three replicates were maintained in each case. Bacterial and fungal colonies were identified and characterized based on their morphological, cultural and reproductive characteristics on the growth media.

Isolation, Quantification and Root Colonization of VAM Spores: Wet sieving and decanting technique [16, 17] was followed to isolate VAM spores. For this, 50 gm of soil was suspended in 500 ml water and decanted by using a series of sieves. Spores retained on the mesh were recovered by repeated washing and transferred to whatman no. 01 filter paper in a petridish and observed under stereo-binocular microscope. The isolated spores were identified using the keys of various mycologist [18, 19, 20, 21,2 2]. Websites, www.mycorrhiza.com,www.ffp.csiro.aug.utk/research/mycorrhiza/intro,http://zor.zut.e .pl/Glomeromycota/index.html are also used for identification.

Mycorrhizal Quantification: A modified method was used for quantitative estimation of VAM spores. The Whatman filter paper was divided into many small sectors and total numbers of spores were counted by adding the number of spores present in each sector under stereo-binocular microscope [23].

Growth Media, Isolation and Culture Conditions for Rhizospheric Fungi: Potato dextrose agar (PDA) was used for the isolation of soil fungi using dilution plate technique [24] and 10^5 dilutions. The media were supplemented with 50µg/ml of streptomycin sulphate to prevent bacterial growth. Fungi were grown at 25±1 °C for 5 days. Three replicates were maintained in each case. Pure colonies were transferred to PDA slants overlaid with mineral oil and stored at 4 °C for further identification. Fungi were characterized

based on their cultural, morphological and spore characteristics and identified by consulting various taxonomic monographs [25, 26, 27, 28]. The fungi that did not produce spores were characterized as mycelia sterile and those showing no diagnostic morphological characters were included under unidentified strains.

Isolation and Characterization of Soil Bacteria: Isolation of soil bacteria was done on Nutrient agar (NA) using dilution plate technique of Johnson and Curl, (1972) and 10^6 dilutions. The bacterial population was estimated by growing them at 30±1 °C for 48 h. Three replicates were maintained in each case. Pure cultures of bacteria were preserved at 4 ºC in NA slants after observing the abundance of bacterial growth, pigmentation and optical characteristics. For long-term storage, isolates were kept in 15% (v/v) glycerol in NB at –20 ºC. Bacterial morphological characters like shape, size, texture, surface, growth, elevation, margin type, consistency, pigmentation, rate of growth *etc*., as well as physiological and biochemical characteristics were examined in accordance with standard monographs [29, 30]. A modified gram staining method [31] was followed to differentiate the Gram-positive bacterial isolates from Gram-negative strains.

Production of *In-vitro* Cultures and Mass Multiplication of Selected Indigenous and Putative Bioagents/Microsymbionts for Bio-Inoculation of Target Plant Species:

Mycorrhizal Mass Multiplication: The mycorrhizal inoculum production was carried out using soil funnel technique [32]. Dominant single and efficient AM spore can be used for mass production here. Sorghum, maize, gram and wheat were selected as the best host for starter culture of inoculum production. In the technique, earthen funnels were taken for the germination of seeds .Observation was continued until the root of the seedlings touched the inoculum of AM fungi. The seedlings were raised up to 30 days in the earthen funnels containing sterilized sand and soil at the ratio of 1:3. In the present investigation, 40 g sand was taken against 120 g of soil. The experiment was repeated up to 45-90 days and AM spores were collected by wet sieving and decanting technique [33, 34]. The spores of *Glomus* sp. and *Acaulospora* sp. were utilized in the present investigation for mass multiplication using hosts like *Zea mays* L. (maize) and *Cicer arietinum* L. (chickpea) in bigger earthen pots.

Pot Culture: Mass multiplication of dominant AM spores like *Glomus* sp. and *Acaulospora* sp. was carried out using different hosts and substrates in pots. Maize (*Zea mays* L.) and chick pea (*Cicer arietinum* L.) was observed as best hosts for pot cultures of mycorrhizal spores. Sand and soil (1:3) was used as substrates for pot cultures. The pot cultures were maintained for several days. The pots were supplemented with Hoagland solution once in a fortnight. However, KH_2PO_4 was removed from the original solution to observe the effect. The soil containing mycorrhizal spores, mycelium and colonized roots was used, further, to inoculate seedlings and to prepare other pot cultures.

Field Culture: The test inocula were mass multiplied in field conditions by preparing standard size beds on thin polyethylene sheet (0.5 mm). Care was taken so that no contamination occurred to the inocula. The experiment was repeated for maintaining the inocula cultures more viable for further experimentation.

Mass Multiplication of Selected Fungal Isolate: *Trichoderma*, being a potent fungal biocontrol agent is known for its antagonistic action against a range of plant pathogens [35, 36]. Different organic media like neem cake, coir pith, farmyard manure, decomposed coffee pulp are being used for *Trichoderma* multiplication [37]. However, standard method [38] was employed in the present investigation for the mass multiplication of *Trichoderma* spp.

Collection of Soil Samples and Isolation of Compost Fungal Activator: The soil samples were collected from different locations of Assam and Meghalaya of N. E. India. *Trichoderma harzianum*, the fungal stain was isolated from the collected soil samples by using serial dilution plate method on PDA medium. The inoculated plates were incubated at 30 ^oC for 4 days. Fungal colonies were purified by streak plate method on agar slants and incubated at 30 ^oC for 7-8 days. Green conidia forming fungal bodies were selected and microscopic observation was made for fungal identification.

In the present investigation, the fungus was identified as *Trichoderma harzianum* (Isolate no. TH-13). The identified fungal isolate was maintained on PDA slants is retained with Mycology and Soil Microbiology Laboratory, (RFRI), Jorhat, Assam, India, for further study and analysis.

Preparation of Solid Substrate: Saw dust of *Shorea robusta* Gaertn. was used in the present investigation for solid substrate preparation. For this, saw dusts were shade dried and mixed well with wheat bran by adding SDW in the ratio of 3:1:4 w/w where, 03 parts of wheat bran is mixed with 01 parts of saw-dust and 04 parts of water. The moisture of the mixture was maintained up to 50–60%. Autoclaving is made to sterilize the substrate properly.

Mass Multiplication of *Trichoderma harzianum:* *Trichoderma harzianum* was grown on synthetic PDA medium (SRL, India) for 7-8 days and incubated at 27–30^{o} ± 1^{o}C. The inoculum was kept in BOD incubator (Labotech, BDI-55 make, India) for 10–12 days for maximum growth and sporulation. The inoculum containing medium was cut into small discs and put in flasks containing wheat bran and saw-dust medium in the ratio of 3: 1: 4 w/w for mass multiplication of *Trichoderma harzianum*. Approximately 50 g substrate was put in 500 ml conical flasks followed by inoculation with 5 mm mycelial mat. It was then incubated at 28 $^{\circ}$C for 7–10 days. The target bioagent in the form of substrate inoculum was applied at the time of sowing of target seedlings in the nursery of RFRI, Jorhat, Assam N. E. India.

Mass Multiplication of Selected Bacterial Isolate/s:

Isolation of Putative Bacterial Bioagent: *Pseudomonas* sp. was isolated from the rhizosphere of target plant species of the selected study sites using NB medium. After incubation, individual colonies were transferred to fresh NA slants. Pure colonies were stored at 4 °C for further use. Colony characteristics were observed regularly. Individual colonies were examined for typical shape, size, structure and pigmentation.

Mass Multiplication of *Pseudomonas* sp.: Pure colonies of selected bacterial isolates were streaked with the help of a sterile loop and dipped in a freshly prepared NB and incubated at an orbital shaking incubator for 2 days. After the incubation period is over, the clear medium appears as frothy and slightly pale colour indicating the growth of *Pseudomonas* sp. (Isolate PS-I). The mass multiplied liquid media were used for further inoculation experiments.

Investigation on the Efficacy of Selected Indigenous Putative Bioagents/Microsymbionts on the Target Plant Species in Nursery and Their Role in its Establishment and Conservation: Randomized Block Design (RBD) was made in the nursery of RFRI, Jorhat Assam, India. Selected bioagent was inoculated on the seedling stock of target plant species, *Elaeagnus latifolia* L. Three different inoculation procedures such as single, double and synergistic were adopted in the present investigation. Three replicates were maintained for each treatment. A treatment without any inoculum served as control. Root trainer (30cm×50m) containing 500 g of substrates like sand and soil at the ratio of 1:2 was used for each treatment along with its replication. Inoculum was applied close to the rhizosphere of the seedlings (at the depth of 5-10 cm).

In the first set of experiment, eight treatments were undertaken consisting of mono, dual and tri-inoculums of the selected indigenous bioagents consisting of *Glomus mosseae*, *Pseudomonas putida* and *Trichoderma harzianum*. For convenience, the various synergistic treatments are labeled as follows:

T1 = Control (No inoculation).

T2 = Treatment/Inoculation of A (*Pseudomonas putida*).

T3 = Treatment/Inoculation of B (*Trichoderma harzianum*).

T4 = Treatment/Inoculation of C (*Glomus mosseae*).

T5 = Treatment/Inoculation (combination of A+B).

T6 = Treatment/Inoculation (combination of B+C).

T7 = Treatment/Inoculation (combination of C+A).

T8 = Treatment/Inoculation (combination of A+B+C).

Qualitative and quantitative estimation of the secondary metabolites in *Elaegnus latifolia* L. was carried out compounds like alkaloids, tannins, flavonoids and lignin. Effect of microbial inoculation on nutrient contents like protein, sugar and carbohydrates, IAA, pigments such as chlorophyll, anthocyanins, carotenoids content in the target plant was also enumerated.

Qualitative Analysis of Phytochemicals:

Extraction of the Plant for Phytochemical Screening: Fresh plant materials were washed in tap water and dried using blotting paper. The plant materials were then air and shade dried successively for two weeks and powdered using mortar. The air-dried and powered leaves of about 50 g were extracted with 250 ml of petroleum ether, ethyl acetate, methanol, chloroform, acetone, benzene and water by using Soxhlet extractor for 8 hour up to the boiling temperature of the solvent used. The aqueous extracts were filtered using Whattman No.1 filter paper and concentrated in vacuum at 4 °C using rotary evaporator. The residues, so, obtained were stored in a freezer at -70 ^{o}C for future analysis. Phytochemical screenings of the extracts were carried out using standard protocols.

Test for Tannins ($FeCl_3$ Test): Appearance of a deep blue-black colour after addition of 5% $Fecl_3$ solution to crude extract indicates the presence of tannin.

Test for Flavonoids:

Shinoda Test: The test was performed by mixing few fragmements of Mg ribbons with the plant extract. Conc. Hydrochloric acid (HCL) was added to it drop wise. Appearance of pink, scarlet colour after a few minutes indicates the presence of flavonoids.

Zinc Hydrochloride Test: This test is done with mixtures of zinc dust and conc. HCL. Red colour indicates a positive reaction.

Test for Lignin:

Wiesner Test: This test is performed by using a piece of softwood or of low-grade paper (phloroglucinol) dipped in alcohol and conc. HCL. Development of a brilliant red color indicates the presence of aldehyde group in lignin.

Detection of Alkaloids: Crude extracts were dissolved individually in dilute HCL and filtered. Two confirmation tests such as Mayer's and Hager's tests were performed to detect the presence of alkaloids.

Mayer's Test: Filtrates were treated with Mayer's reagent (potassium mercuric iodide). Yellow coloured precipitate indicates the presence of alkaloids.

Hager's Test: Hager's reagent was added to the 2-3 ml of filtrate. Yellow ppt. indicated the presence of alkaloids.

Detection of Carbohydrates: Extracts were dissolved individually in 5 ml SDW and filtered. The filtrate was used to test the presence of carbohydrates.

Fehling's Test: Filtrate was hydrolyzed with dilute HCL and neutralized with alkali. Samples were then treated with Fehling's solution A and B. Red coloured precipitate indicates the presence of reducing sugar.

Detection of Glycosides: Sample extracts were hydrolyzed with dilute HCl and subjected to test for glycosides.

Borntrager's Test: To 2.0 ml of filtrate hydrolysate, 3.0 ml of chloroform was added and shaked well. Chloroform layer was separated, and 10% ammonia solution was added to it. Pink color indicated the presence of glycosides.

Detection of Steroids and Terpenoids: To 1.0 ml of methanol plant extract, 1.0 ml chloroform followed by 2-3 ml of acetic anhydride was added and mixed well. 1-2 drops of conc. H_2SO_4 was added to it. Presence of dark green colour of the solution indicated the presence of steroids while pink, red colour of the solution indicated the presence of terpenoids in the sample.

Detection of Saponins:

Froth Test: Extracts were diluted with SDW, and volume was made up to 20 ml. It was then shaked in a graduated cylinder for 15 minutes. Formation

of foam layer (up to 1 cm) indicated the presence of saponins.

Foam Test: 0.5 g of plant extracts were shaked with 2.0 ml of water. Persistence of foam for about 10 minutes indicated that the presence of saponins.

Detection of Phenols:
Ferric Chloride test ($Fecl_3$): Crude extracts were treated with 3-4 drops of $Fecl_3$ solution. Bluish black colour indicated the presence of phenols.

Detection of Proteins and Amino Acids:
Xanthoproteic Test: The extracts were treated with few drops of conc. nitric acid (HNO_3). Formation of yellow colour indicated the presence of proteins.

Detection of Flavonoids: To the methanolic extract, 10% NaOH followed by dilute HCl was added. Change in colour from yellow to colourless indicates a positive reaction.

Quantitative Analysis of Phytochemicals:
Determination of Total Phenolic Compounds: Total Phenolic Content (TPC) was determined by Folin-Ciocalteu's colorimetric method [39]. For this, 100 mg of the crude extract of the sample was weighed and dissolved in 100 ml triple distilled water (TDW). 1.0 ml of this solution was transferred to a test tube where 0.5 ml 2N of Folin-Ciocalteu reagent and 1.5 ml of 20% of Na_2CO_3 solution was added and finally the volume was adjusted to 8.0 ml with TDW. Vigorous shaking is made and finally it was allowed to stand for 2 hours. Absorbance was estimated spectrophotometrically at 765 nm. The data were used to estimate the total phenolic content using a standard calibration curve obtained from gallic acid at different concentrations.

Determination of Total Flavonoids: The method is based on the formation of flavonoid - aluminium complex that has an optimum absorbance at 415nm. For this, 100µl methanolic plant extract (10 mg/ml) was mixed with 100 µl of 20 % aluminum trichloride where, a drop of acetic acid was added. It was then diluted with methanol and the volume was made up to 5.0 ml. Absorbance was read at 415 nm after 40 minutes. Blank samples were prepared using 100 ml of the plant extract mixed in one drop of acetic acid and diluted to 5.0 ml with methanol. Absorption of standard routine solution (0.5 mg/ml) in methanol was measured. All calculations were carried out in triplicates.

Determination of Total Alkaloids: 5 g of the sample was weighed in a 250 ml beaker where 200 ml of 10% acetic acid in ethanol was added. The mixture was covered and allowed to stand for 4 hours. It was then filtered and concentrated on water bath to 1/4 of the original volume. Conc. ammonium hydroxide (NH_4OH) was added dropwise to the extract until precipitation was completed. The whole solution was allowed to settle and precipitate was collected and washed with dilute NH_4OH and filtered. The residue, so obtained, was dried and weighed. It was considered as the alkaloids present in the sample.

Determination of Total Tannins: To determine the total tannins present, 500 mg of the sample was weighed into a 50 ml plastic bottle. 50 ml of SDW was added to it and shaken for 1 hour and later filtered into a 50 ml volumetric flask. 5.0 ml of the filtrate was pipetted out into a test tube and mixed with 2.0 ml of 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min.

DPPH (1, 1-Diphenyl -2-picrylhydrazyl) Method to Measure free Radical Scavenging Activity: The antioxidant activities were determined using DPPH (Sigma-Aldrich, Germany; M.W.394.32M) as a free radical. For this, 1µg/ml solution of the methanolic plant extract was prepared along with 6x 10-5 mol/L DPPH in methanol. 0.1 ml of sample extract was added to 3.9 ml of DPPH solution. Absorbance was read at 517nm at 1 min interval up to 15 minutes. A blank sample was prepared using methanol and DPPH solution that serves as control. Ascorbic acid was used as standard. Three replicates were maintained at each case. Free radical scavenging activity was measured using the following formula:

Percentage (%) DPPH scavenging activity = (Absorbance of control - Absorbance of test sample) / Absorbance of control × 100

Hydrogen Peroxide Scavenging Activity: The ability of *E. latifolia* L. extracts to scavenge hydrogen peroxide (H_2O_2) [40]. For this, a solution of 40 ml H_2O_2 was prepared in phosphate buffer (pH

7.4). 100 µg/ml plant extract in distilled water were added to it (0.6 ml, H_2O_2 of 40 mM). Absorbance was read at 230 nm after 10 minutes. A solution containing the phosphate buffer without H_2O_2 serves as control. Percentage of H_2O_2 scavenging activity of *E. latifolia* L. extract and standard were calculated using the following formula:

$$\% \text{ Inhibition} = (AC\text{-}AS) / AC)/AC] \times 100$$

Where, AC = the absorbance of the control, AS = the absorbance in the presence of the sample extracts or standards.

Determination of Chlorophyll: Chlorophyll content was estimated using Arnon's (1949) method [41]. For this, 100 mg leaf sample was homogenized with 10 ml of 80% pre-chilled acetone. Extract was centrifuged at 3000 rpm for 10 minutes. Supernatant was collected and absorbance was read spectrophotometrically at 645 and 663 nm respectively. Total chlorophyll content was measured using following formula**.**

$$\text{Total Chl} = 20.2 (A645) + 8.02 (A\ 663) \times V/1000 \times W \times 10$$

Where, A = OD at specific nm, V = Final volume of plant extract in 80% acetone, W = Fresh weight of leaf tissue used.

Determination of Anthocyanin: The anthocyanin content was determined in accordance with Swain and Hillis, (1959)[42]. 100 mg fresh leaves were ground with 10 ml of ethanol and filtered through Whatman No.1 filter paper. 1.0 ml of the filtrate was treated with methanolic HCl. with 1.0 ml peroxide reagent was added to the mixture. It was then kept in dark for 15 minutes and absorbance was read at 525 nm. Anthocyanin content was represented by optical density (OD) value (A525)/gram of leaf tissue.

Estimation of Protein Content: The protein content was estimated using standard protocol [43]. For this, 1.0 gm of leaf sample was ground with 5.0 ml of phosphate buffer (pH 7) and centrifuged at 3000 rpm for 20 minutes. 3.0 ml of the extract was then mixed with equal volume of 20% trichloroacetic acid and put in water bath for 20 minutes and centrifuged at 3000 rpm for 20 minutes.

The pellet, so obtained, was collected and washed with 6 ml acetone and centrifuged. It was dissolved in 5.0 ml of 0.1 N NaOH and shaked well. 0.5 ml of folin was added to it and incubated for 30 minutes in dark. Absorbance was read at 660 nm. Bovine serum albumin was used as standard.

Determination of Total Soluble Sugars: The amount of total soluble sugar in the leaf extract was determined using Anthrone method [44]. For this, 100 mg of leaf sample was ground with 10 ml of distilled water. 2.0 ml of 10% trichloroacetic acid was added to it and centrifuged at 3000 rpm for 5 minutes. 4.0 ml of anthrone reagent was added to 1.0 ml of the supernatant in test tubes. The tubes were kept in water bath after carefully covering their mouth with glass marbles for 10 minutes. The content was cooled, and absorbance was read at 625 nm. The amount of sugar was determined using glucose as standard.

RESULTS: Qualitative estimation of the secondary metabolites in the said species was done and the following compounds were present *viz.* alkaloids, tannins, flavonoids, lignin **Table 1.**

TABLE 1: QUALITATIVE ANALYSIS OF PHYTOCHEMICALS

Phytochemical	Phenol	Flavonoid	Lignin	Alkaloid	Steroids	Terpenoids	Carbohydrates	Glycosides	Saponin	Proteins	Aminoacids
Status	++	++	+	++	+	+	++	+	+	++	+

+= Present, ++= Present in high amount.

Phenolic compounds are a class of antioxidant agents which act as free radical terminators and their bioactivities may be related to their abilities to chelate metals, inhibit lipoxygenase and scavenge free radicals. The amount of total phenol in the plant samples was determined with the Folin-Ciocalteu reagent. Catechol was used as a standard compound and the total phenols were expressed as

mg/g catechol equivalent using the standard standard curve equation: y = 0.0001x + 0.026, R^2= 0.951, Where y is absorbance at 625 nm and x is total phenolic content in the extracts of *E. latifolia* **Fig. 2**. **Table 1** depicts the standard reading of Phenols (catechol in µg/ml). **Fig. 1** depicts the standard curve for the determination of phenol content of the inoculated seedlings. The total phenol varied from 107±0.05 to 173 ± 0.025 mg/g in the extracts of the treated seedlings in the first set of experiment **Fig. 3.** The maximum phenolic content was obtained in the ethanolic extracts of T3 (173 ± 0.025 mg/g) treated seedlings while the least amount of phenol was found in T1 treatment i.e. Control (107±0.05 mg/g).

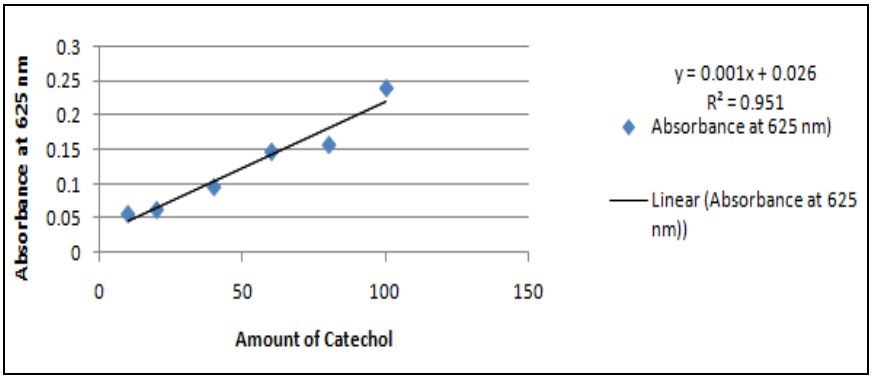

FIG. 2: STANDARD CURVE FOR THE DETERMINATION OF PHENOL

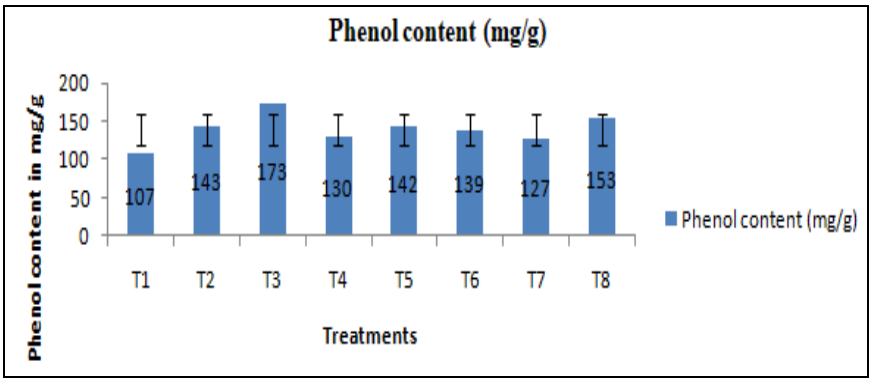

FIG. 3: PHENOL CONTENT (MG/G). T1= Control, T2 = Inoculation of A (*Pseudomonas putida*), T3= Inoculation of B (*Trichoderma harzianum*), T4 =Inoculation of C (*Glomus mosseae*), T5 = Combination of A+B, T6= Combination of B+C, T7= Combination of C+A, T8= Combination of A+B+C

The amount of total flavonoid was determined with the Quercetin reagent. Quercetin was used as a standard compound and the total flavonoids were expressed as mg/g Quercetin equivalent using the standard curve equation: y = 0.096x +0.060, R^2= 0.991, Where y is absorbance at 510 nm and x is total flavonoid content in the extracts of treated seedlings of *E. latifolia* expressed in mg/gm. **Fig. 4** depicts the standard curve for the determination of flavonoid content of the inoculated seedlings. The total flavonoid varied from 28.44±0.01 to 85.42±0.015 mg/g in the extracts of the treated seedlings **Fig. 5**. The maximum flavonoid content was obtained in the ethanolic extracts of T8 (85.42±0.015 mg/g) treated seedlings while the least amount of flavonoid was found in T1 treatment i.e. Control (28.13 ±30 mg/g). The free radical scavenging activity of root extracts of *E. latifoliia* root was studied by its ability to reduce the DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH, can react with it and thereby bleach the DPPH absorption. DPPH is a purple color dye having absorption maxima of 517 nm and upon reaction with a hydrogen donor the purple color fades or disappears due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance extracts exhibited considerable DPPH free radical scavenging activity as indicated by their IC_{50} values. IC_{50} Indicate the potency of scavenging activity. Standard ascorbic acid IC_{50} of 5.45 µg/ml. In comparison to standard ascorbic acid, the ethanolic extract of *E. latifolia* root showed an IC_{50} of 11.70 µg/ml respectively.

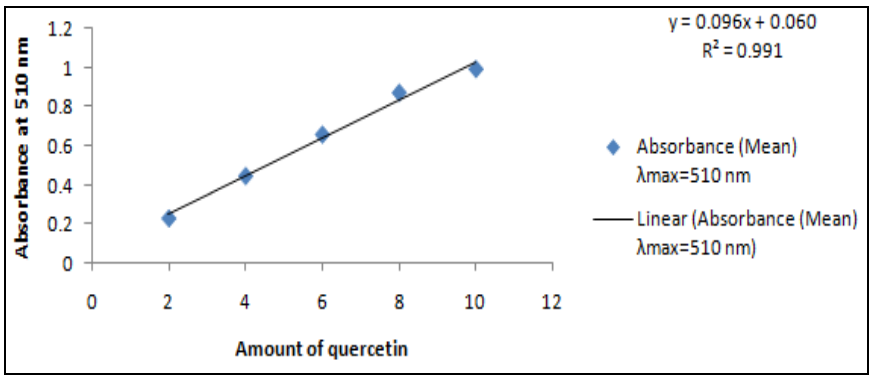

FIG. 4: STANDARD CURVE FOR THE DETERMINATION OF FLAVONOIDS AND FLAVONOL

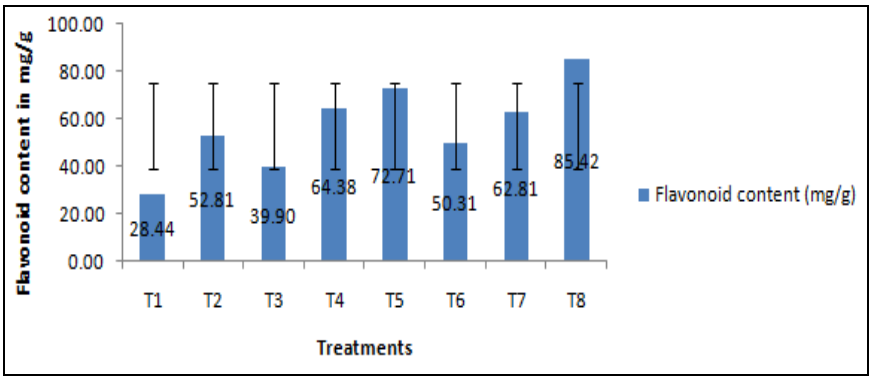

FIG. 5: FLAVONOID CONTENT IN *E. LATIFOLIA* INOCULATED SEEDLINGS (AFTER 240 DAYS OF INOCULATION. T1= Control, T2 = Inoculation of A (Pseudomonas putida), T3= Inoculation of B (Trichoderma harzianum), T4 =Inoculation of C (Glomus mosseae), T5 = Combination of A+B, T6= Combination of B+C, T7= Combination of C+A, T8= Combination of A+B+C

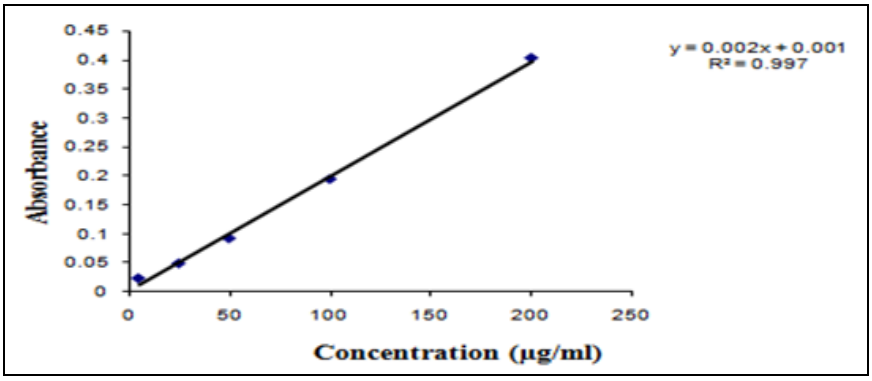

FIG. 6: CALIBRATION CURVE OF ASCORBIC ACID

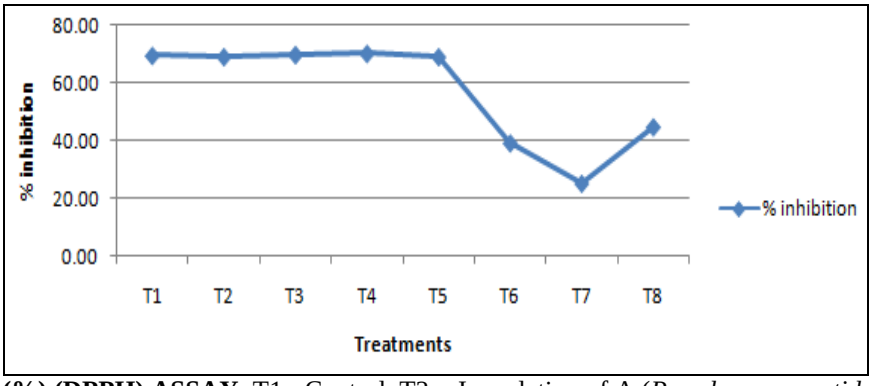

FIG. 7: INHIBITION (%) (DPPH) ASSAY. T1= Control, T2 = Inoculation of A (*Pseudomonas putida*), T3= Inoculation of B (*Trichoderma harzianum*), T4 =Inoculation of C (*Glomus mosseae*), T5 = Combination of A+B, T6= Combination of B+C, T7= Combination of C+A, T8= Combination of A+B+C

Analyses were done to observe the effect of bioinoculation in the free radical scavenging activity of root extracts of *E. latifolia* using DPPH assay. The standard curve is depicted in **Fig. 5**. The results revealed that, the highest % inhibition activity was showed by T4 treatment (95.58 ± 0.02) and the least was found in T5 treatment (76.17 ± 0.03) **Fig. 6.** Hydrogen peroxide, although not a radical species play a role to contribute oxidative stress. The generation of even low levels of H_2O_2 in biological systems may be important. Naturally occurring iron complexes inside the cell believed to react with H_2O_2 *in-vivo* to generate highly reactive hydroxyl radicals and this may be the origin of many of its toxic effects. Scavenging of hydrogen peroxide of different extracts of *E. latifolia* root is presented. The highest % inhibition activity was showed by T4 treatment (70.36± 0.01) and the least was found in T7 treatment (25.04± 0.03) **Fig. 8.**

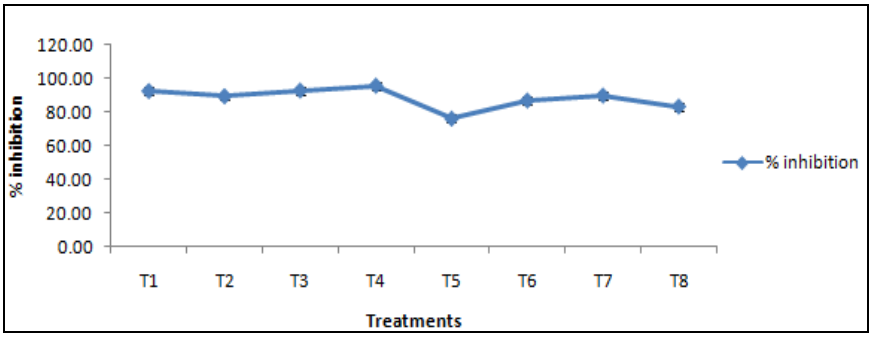

FIG 8: INHIBITION (%) (HYDROGEN PEROXIDE ASSAY). T1= Control, T2 = Inoculation of A (*Pseudomonas putida*), T3= Inoculation of B (*Trichoderma harzianum*), T4 =Inoculation of C (*Glomus mosseae*), T5 = Combination of A+B, T6= Combination of B+C, T7= Combination of C+A, T8= Combination of A+B+C

The amount of total protein content (Plate 1) was determined with the BSA as the standard and were expressed as mg/g BSA equivalent using the standard curve equation:

$y = 0.001x + 0.051$, $R^2 = 0.984$,

Where y is absorbance at 660 nm and x is total protein content in the extracts of treated seedlings of *E. latifolia* expressed in mg/gm.

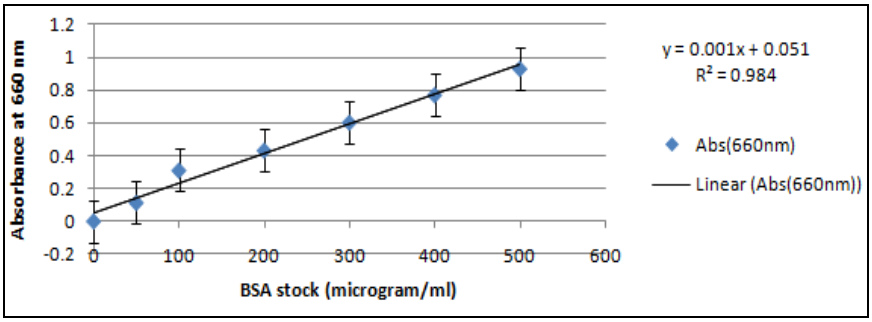

FIG. 9: STANDARD CURVE FOR THE DETERMINATION OF PROTEIN CONTENT

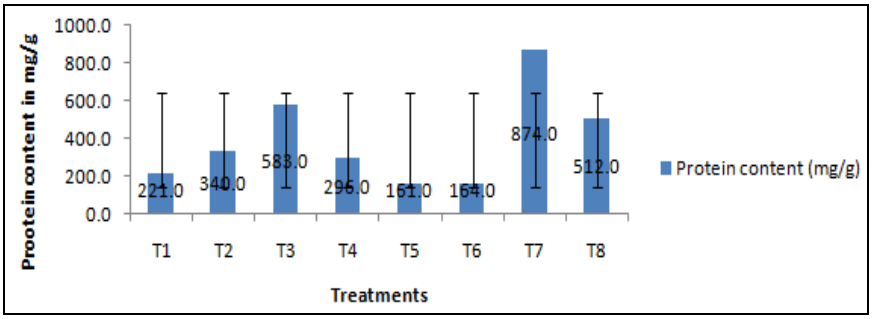

FIG. 10: PROTEIN CONTENT IN *E.LATIFOLIA* INOCULATED SEEDLINGS (AFTER 240 DAYS OF INOCULATION). T1= Control, T2 = Inoculation of A (*Pseudomonas putida*), T3= Inoculation of B (*Trichoderma harzianum*), T4 =Inoculation of C (*Glomus mosseae*), T5 = Combination of A+B, T6= Combination of B+C, T7= Combination of C+A, T8= Combination of A+B+C

Fig. 9 depicts the standard curve for the determination of total protein content of the inoculated seedlings. The maximum total protein content was obtained in the T8 (874±0.30 mg/g) treated seedlings while the least amount of total protein content was found in T5 treatment (161 ±31 mg/g) **Fig. 10.** The effect of bioinoculation in the total soluble sugar content of the inoculated seedlings of *E. latifolia*. The amount of total soluble sugar was determined with the glucose as the standard and were expressed as mg/g BSA equivalent using the standard curve equation:

$$y = 0.241x + 0.070,\ R^2 = 0.959,$$

Where y is absorbance at 625 nm and x is total soluble sugar content in the extracts of treated seedlings of *E. latifolia* expressed in mg/gm. **Fig. 11** depicts the standard curve for the determination of total soluble sugar content of the inoculated seedlings. The maximum total soluble sugar content was obtained in the T1 (10.08±0.47 mg/g) treated seedlings while the least amount of total soluble sugar content was found in T4 treatment (4.42 ±0.52 mg/g) **Fig. 12.**

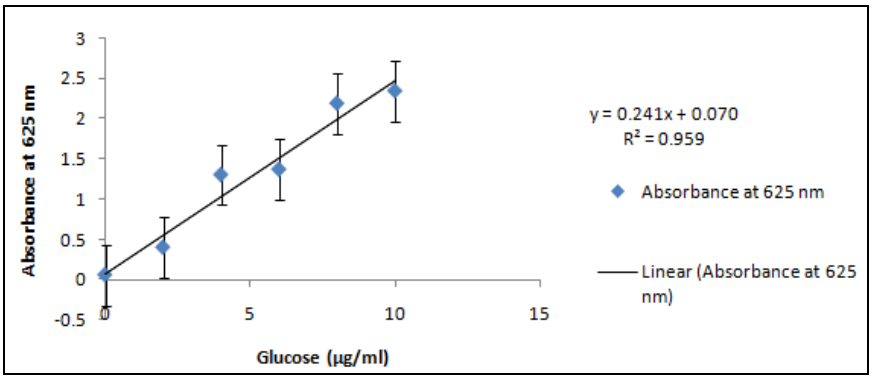

FIG. 11: STANDARD CURVE FOR THE DETERMINATION OF TOTAL SOLUBLE SUGAR

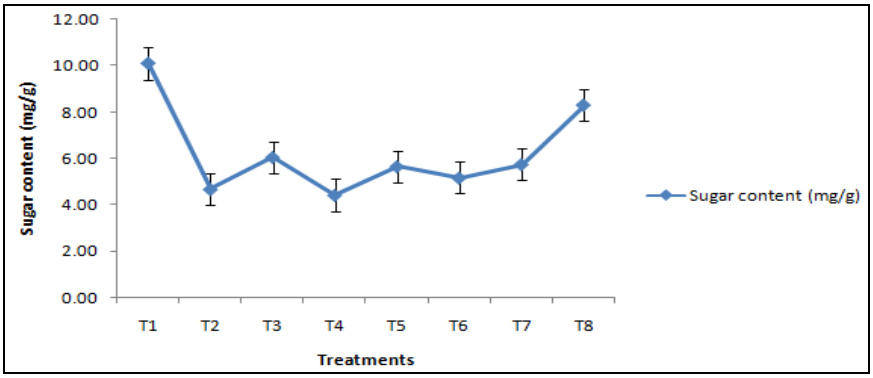

FIG. 12: SUGAR CONTENT IN *E.LATIFOLIA* INOCULATED SEEDLINGS (AFTER 240 DAYS OF INOCULATION). T1= Control, T2 = Inoculation of A (*Pseudomonas putida*), T3= Inoculation of B (*Trichoderma harzianum*), T4 =Inoculation of C (*Glomus mosseae*), T5 = Combination of A+B, T6= Combination of B+C, T7= Combination of C+A, T8= Combination of A+B+C

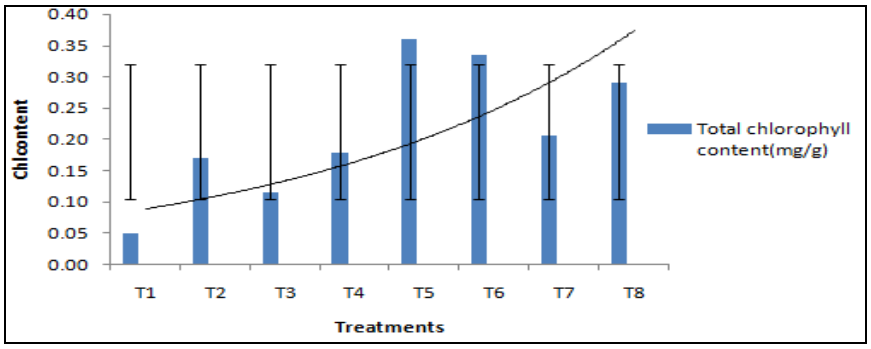

FIG. 13: HISTOGRAM SHOWING THE TOTAL CHLOROPHYLL CONTENT (MG/G) IN THE INOCULATED SEEDLINGS. T1= Control, T2 = Inoculation of A (*Pseudomonas putida*), T3= Inoculation of B (*Trichoderma harzianum*), T4 =Inoculation of C (*Glomus mosseae*), T5 = Combination of A+B, T6= Combination of B+C, T7= Combination of C+A, T8= Combination of A+B+C

Analysis of data on the effect of bioinoculation the total chlorophyll content in the experiment is shown in **Fig. 13**. It is evident that, the maximum total chlorophyll content was observed in T5 (0.36 mg/g) while the least chlorophyll content is found in T1 treatment (0.05 mg/g). **Fig 14** and **Fig. 15** depicts the effect of bioinoculation in the total carotenoid and anthocyanin content in the treated seedlings. The maximum total carotenoid content was observed in T5 (0.015 mg/g) while the least total carotenoid content is found in T1 treatment (0.004 mg/g). The maximum anthocyanin content was observed in T6 and the least in T3.

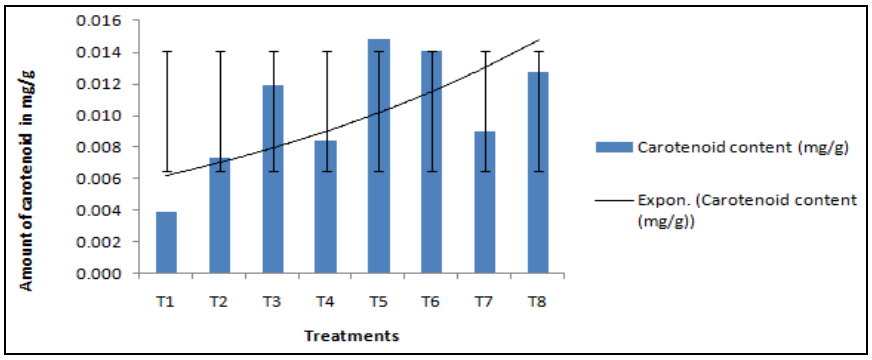

FIG. 14: HISTOGRAM SHOWING THE TOTAL CAROTENOID CONTENT (MG/G) IN THE INOCULATED SEEDLINGS. T1= Control, T2 = Inoculation of A (*Pseudomonas putida*), T3= Inoculation of B (*Trichoderma harzianum*), T4 =Inoculation of C (*Glomus mosseae*), T5 = Combination of A+B, T6= Combination of B+C, T7= Combination of C+A, T8= Combination of A+B+C

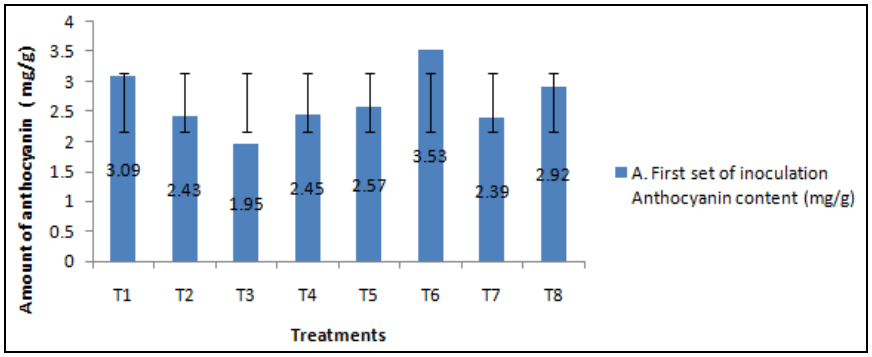

FIG. 15: HISTOGRAM SHOWING TOTAL ANTHOCYANIN CONTENT IN THE INOCULATED SEEDLINGS (FIRST SET OF EXPERIMENT). T1= Control, T2 = Inoculation of A (*Pseudomonas putida*), T3= Inoculation of B (*Trichoderma harzianum*), T4 =Inoculation of C (*Glomus mosseae*), T5 = Combination of A+B, T6= Combination of B+C, T7= Combination of C+A, T8= Combination of A+B+C

DISCUSSION: Significant variations were observed in most of vegetative growth parameters, photosynthetic pigments, carbohydrate, phenolic content, as well as seed yield, yield components, and nutritive value (oil, protein, flavonoid, and phenolic content) of the yielded seeds between three flax cultivars. Sakha-1 cultivar was characterized by highest shoot height, root length, chlorophyll b and polysaccharides, as well as seed and straw yield/fed, protein percentage. The highest significant values of carotenoid, total and soluble carbohydrate, IAA, and phenolic content appeared in linola cultivar [45]. The influence of AMF (*Glomus mosseae*) inoculation, alone or in combination with PGPR (*Bacillus amyloliquefaciens*), on biomass accumulation, morphological characteristics, photosynthetic capacity, and rhizospheric soil enzyme activities of *Elaeagnus angustifolia* L., a typical halophyte in the northwest of China was evaluated [46]. The results indicate that, for one-year-old seedlings of *Elaeagnus angustifolia* L., AMF significantly promoted biomass accumulation in aboveground organs, increased the numbers of leaves and branches, and improved the leaf areas, stem diameters and plant height. AMF-mediated morphological characteristics of aboveground organs favoured light interception and absorption and maximized the capacities for photosynthesis, transpiration, carbon dioxide assimilation and gas exchange of *Elaeagnus angustifolia* L. seedlings in

saline soil. AMF also promoted root growth, modified root architecture, and enhanced soil enzyme activities. *Elaeagnus angustifolia* L. was more responsive to specific inoculation by AMF than by a combination of AMF and PGPR or by solely PGPR in saline soils. Plant roots respond to environmental conditions through the secretion of a wide range of compounds, according to nutritional status and soil conditions [47,48]. This action interferes with the plant-bacteria interaction and is an important factor contributing to the efficiency of the inoculant. Root exudation includes the secretion of ions, free oxygen and water, enzymes, mucilage, and a diverse array of C-containing primary and secondary metabolites [49]. The roots of plants excrete 10-44% of photosynthetically fixed C, which serves as energy source, signaling molecules or antimicrobials for soil microorganisms [50]. The root exudation varies with plant age and genotype, and consequently specific microorganisms respond and interact with different host plants [51, 52, 53]. Thus, inoculants are generally destined to the one specific plant from which the bacterium was isolated.

The well-studied flavonoids also vary with plant age and physiological state when exuded from legume rhizospheres, and induce *nodD* gene expression in rhizobial strains. Nod D is a transcriptional activator of bacterial genes involved in the infection and nodule formation during the establishment of legume-rhizobia symbioses [54]. Similarly to flavonoids, several compounds secreted by roots modulate the relationships between plants and PGPB [55]. *Bacillus subtilis* FB17, for instance, is attracted by L-malic acid, secreted by the roots of *Arabidopsis thaliana* infected with the foliar pathogen *P. syringae* pv *tomato* (Pst DC3000) [56]. Profiles of secreted secondary metabolites, such as phenolic compounds, flavonoids and hydroxycinnamic derivatives, were different in rice cultivars (Nipponbare and Cigalon), according to inoculation with *Azospirillum* 4B and B510 strains. Interestingly, strains 4B and B510 preferentially increased the growth of the cultivar from which they were isolated; however, both strains effectively colonized either at the rhizoplane (4B and B510) or inside roots (B510) [57]. In a similar study, phytochemical investigation of Benzene, Ethyl acetate, Ethanolic and Aqueous extracts of *T. cordifolia* showed the presence of alkaloids, glycoside, flavonoids, phenolic and tannins, carbohydrates, phytosterols and saponin compounds. The maximum possibility is that the presence of flavonoids, tannins and phenolic compound may show anticancer and antioxidant activity of extracts of *T. cordifolia* [58]. The chemical composition, antioxidant, antibacterial activity of isolated oil and methanol extract of *Tridax procumbens* L. were studied. They reported the DPPH antioxidant activity of TPMG oil against its concentration. Gallic acid and ascorbic acid were used as standard references. The result shows that DPPH radical scavenging percentage inhibition varies linearly with concentration from 100 - 500 µg/mL. The IC_{50} value for the essential oil was 18.34 µg/mL. Significant antioxidant activity at $P < 0.05$. TPMG oil showed an antioxidant activity that could prevent and slow the progression of aging in various diseases associated with oxidative stress [59].

Likewise, a small sample of *Aloe vera* and *Clitoria ternatea's* ethanolic, methanolic, and watery extracts were put through a phytochemical test to check alkaloids, tannins, carbohydrates, saponins, phenols, and flavonoids, and steroid and glycosides, as well as a physical chemical test to check the extractive value and ash value. The results of the phytochemical examination revealed phenols, steroids, glycosides, alkaloids, saponins, tannins, flavonoids, and tannins were all present [60].

CONCLUSION: The medicinal plants appear to be rich in secondary metabolites, widely used in traditional medicine to combat and cure various ailments. The anti-inflammatory, antispasmodic, analgesic and diuretic can be attributed to their high alkaloids, phenols, tannins and flavonoids. Exploitation of these pharmacological properties involves further investigation of these active ingredients by implementation of techniques like extraction, purification, separation, crystallization and identification.

ACKNOWLEDGEMENTS: Nil

CONFLICTS OF INTEREST: There is no conflict of interest.

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