Elucidating the Effects of Organic Amendment and Nitrogen Source on Flavonoids and Nitrogen Fixation in *Arachis Hypogaea*

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October 2021

Thesis for the award of MS Degree in Plant Biotechnology,

National University of Sciences & Technology, Islamabad.

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OCTOBER,2021

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Dedication

"My dissertation is dedicated to my loving mother and incredible brothers who supported me and believed in me."

Acknowledgement

All praises for **ALMIGHTY ALLAH**, who is the most magnificent and the ultimate source of knowledge. I am most grateful to Him, for the mental abilities, strengths, blessings, and wisdom that He bestowed to me. All respects are for his **HOLY PROPHET HAZRAT MUHAMMAD** (Peace Be Upon Him) who enabled me to recognize my creator and give me knowledge about Allah. My mother and brother are my inspiration who never let me give up at any cost and support me to their fullest.

I would like to extend my gratitude for **Principal ASAB**, **Dr. Hussnain A Janjua**, **HOD Dr. Muhammad Faraz Bhatti** for providing working environment. I would like to acknowledge my supervisor **Dr. Rabia Amir** for providing the facilities and encouraging atmosphere for scientific work. The confidence I acquired was all due to her constant support. I am grateful to the faculty members of **ASAB, NUST** for their **support** and cooperation.

My deepest gratitude to my respected supervisor **Dr. Rabia Amir,** Co-supervisor **Dr. Ghulam Haider**, my guidance and examination committee **Dr. Muhammad Qasim Hayat, Dr. Faiza Munir** who helped me during my thesis work. The good combination of support, advice, guidance, and friendly attitude made this time challenging and enjoyable. I felt fortunate to work under their supervision and who responded to my questions so promptly without their guidance this thesis would not have been accepted.

I also acknowledge my **mother and brothers** for their love, support and encouragement over the years. I thanks to them for understanding and believing me.

Special thanks to my seniors who provided a constant guidance and support during the research phase and thesis completion. I would also like to acknowledge my lab fellows **Kiran Khurshid** and **Anum Akram** for being compassionate and helping person. I wish best of luck for their future. In the end I would also like to thank my **Lab Fellows** for their help, company and pleasant discussions.

Ahmad Ali

Abbreviations

cDNA	copy of DNA
ESTs	Expressed sequence tags
NCBI	National Centre for Biotechnology Information
MEGA	Molecular Evolutionary Genetic Analysis
ORF	Open Reading Frame
CDS	Coding Sequences
BED	Browser Extensive Data
Aa	Amino acid
NIG	Nod-Inducing genes
GC-MS	Gas Chromatography-Mass Spectrometry
PCR	Polymerase Chain Reaction
Chr	Chromosome
ERN1	Endoplasmic Reticulum to Nucleus Signaling 1
NIN	Nodule Inception
NSP2	Non-Structural Protein 2
NFR5	Nod Factor Receptor 5
BNF	Biological nitrogen fixation
GRAVY	Grand average of hydropathicity
Ka	Non -synonymous
Ks	Synonymous
NJ	Neighbor joining

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Abstract

Biological nitrogen fixation plays a crucial role in the fixation of atmospheric nitrogen. Process of BNF involves flavonoids exudation from the root of leguminous plants, perception of flavonoids on receptors of plant growth-promoting rhizobia, entry of rhizobia in the roots, formation of infectious thread, nod genes induction, and nodules organogenesis in roots. Nodules are the sites for the fixation of atmospheric nitrogen. Identification and characterization of genes in the process of nodulation have not been achieved in the past. In this study, genome-wide analysis of genes involved in the nodulation process has been performed which consists of phylogenetic analysis, conserved motif analysis, gene structure prediction, conserved domain identification, subcellular localization, promoter region prediction, and cis-acting element identification. Homology study identified and characterized a total of 15 genes that revealed functional and structure similarity with few amino acid differences at a scarce position. These genes are widely distributed in the genome of Arachis hypogaea on 10 chromosomes among 20 chromosomes. In-vitro experiment on a golden variety of peanut plants identifies the effect of urea fertilizer with and without biochar on biological nitrogen fixation and plant growth and development. Four different treatments (T1 = control, T2 = Urea, T3 = Biochar, T4 = Urea + Biochar) are included in experimental design. Phenotypic analysis revealed that nitrogen fertilizer with and without biochar is not affecting plant growth and development, however, chlorophyll analysis suggested that urea fertilizer is directly proportional to chlorophyll content. Identification and characterization of nodulation genes lay down the foundation for the improvement of the biological nitrogen fixation process in the future. Moreover, it is proven from the experiment that nitrogen fertilizer (urea) usage in a leguminous crop field is a waste of resources which is environmentally friendly results with high

Chapter 1: Introduction

1.1. Arachis Hypogaea

Arachis hypogaea is a legume crop of the Fabaceae family also known as Leguminosae and is a distinctive variety of genus *Arachis.* Arachis hypogaea are widely distributed across sub-tropical and tropical regions and have a long cycle, alternative reproductive and vegetative side stem, and flowers absence on the central stem. Length of the Peanut plant is measured about 30-35 cm. leaves are pinnate and alternative having four leaflets with none at the terminal and having 1-7 cm length and 1-3 cm width. The flower of the peanut plant lasts for one day with bright yellow color and stood in an axillary cluster above ground. The ovary of the plant is an elongated floral cup at the base of the flower. At the base of the flower, a short thick stem appears after pollination, grows in the downward direction, and enters the soil, termed gynophore (Lukaniuk et al., 2011).

The seed of the peanut is covered with a thin brown coat. Roots are well-settled taproots along with several lateral roots. The root in the peanut plant is a well-developed taproot with several lateral roots extending several inches into the ground and has nodules for biological nitrogen fixation. Well-drained sandy and light loams having pH 5.9-7 is best for Peanut growth but can also grow in heavier soils (Lukaniuk et al., 2011). Atmospheric nitrogen fixation ability is present in Peanut due to nodulation which decreases the usage of industrial nitrogen fertilizer and increases the fertility of the soil effectivity. Nitrogen fixation ability makes peanut active crop in crop rotation. Crop rotation reduces the risk of diseases and pest attacks and enhances the yield of the Peanut crop. Despite crop rotation, the soil must contain a moderate amount of nutrients like calcium, phosphorus, magnesium, potassium, and other micronutrients. Warm weather and about 350 mm (14 inches) groundwater level is required for Peanut plant growth however can provide a high yield at 500 mm (20 inches) of groundwater level (Jauron, et al., 1997).

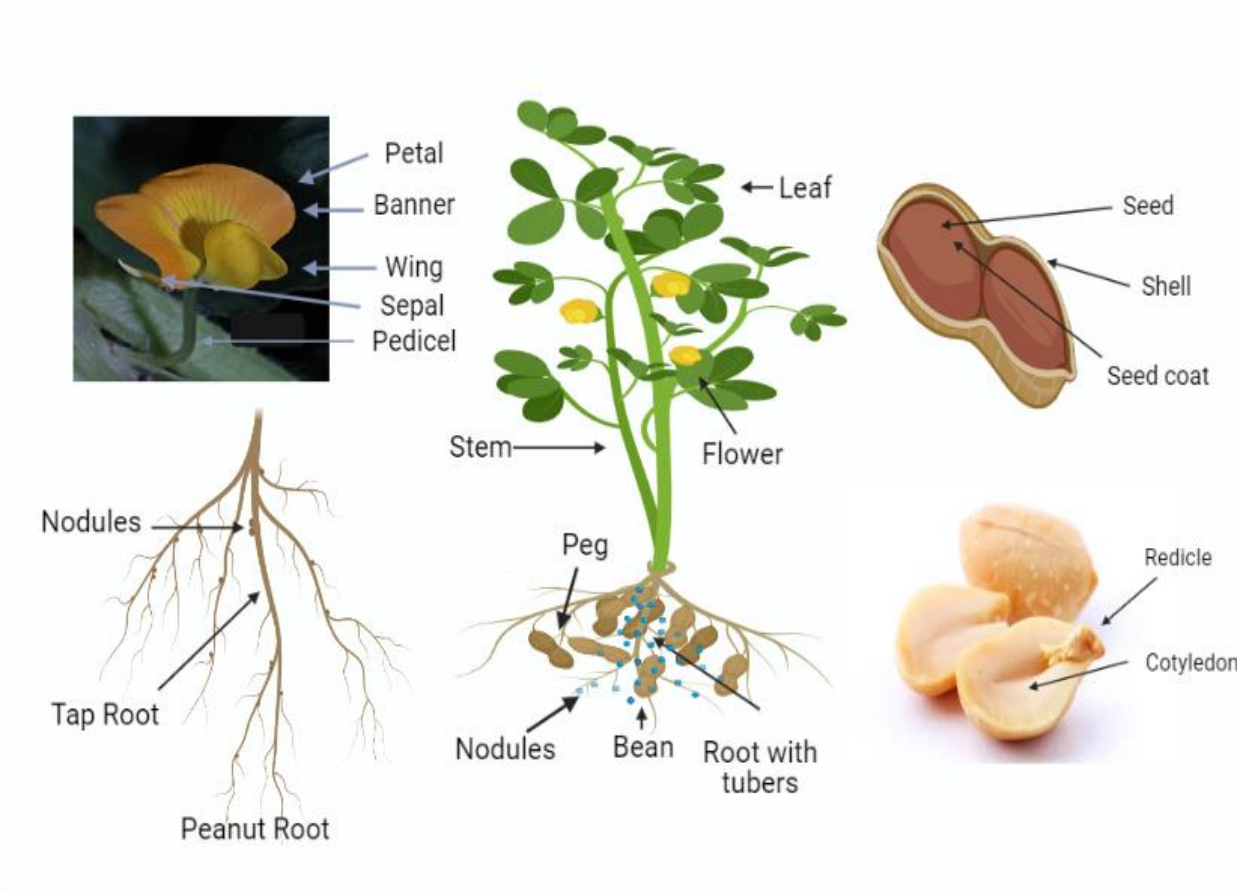

Fig: 1 Mature Peanut (*Arachis hypogaea*) plant with fully developed pods. The pods develop underground after fertilization and contain fertilized seeds. A single pod can contain from 1 to 5 seeds.

1.2. Economic Importance and Uses

Worldwide Peanut has cultivated in around 100 countries on about 24 million hectares area with production of 38 million tons as a legume crop and source of food, oil, and cattle feed. Peanut production rate and cultivation area increased to 0.1% and 0.3% respectively (Birthal et al., 2010). Rain feed lands of Asia and Africa alone produce 95% of peanuts in the world as this crop is a cash crop for farmers living in such regions. This crop is also cultivated for food for the families of farmers and branches of Peanut are used for cattle feeding.

Peanut is an essential source of oil and protein which account for 48-50% and 25-28% of the total content, respectively. Its kernels produce energy of about 564 kcal per 100 g (Jambunathan.

1991). Despite that many beneficial compounds like antioxidants, vitamins, monosaturated fatty acids, and minerals are constituents of Peanut. Resveratrol and p-coumaric are the antioxidants present in peanuts. Peanut also contains vitamin E and many B-complex groups of pantothenic acid, folates, thiamin, vitamin B-6, and niacin. Compounds like flavonoids, polyphenols, and isoflavones which are biologically important are present in peanuts. Groundnut's high nutritional content shows that it might be used in combating food security. Thousands of malnourished children's lives were saved by a therapeutic food Plumpy nut in Niger (UNICEF, 2007). Globally, 60% of total peanut is crushed to get edible oil, and the remaining 40% is used for sowing in the next season and consumed as food (Birthal et al., 2010). Peanut oil is an excellent medium for cooking due to its high smoking point (Singh and Diwakar, 1993).

Peanut shells are used in the fertilizer as fillers and starting raw material for manufacturing boards. This shell also serves as feed for cattle as it contains 8-15% protein, 1-3% lipids, 9-17% minerals, and 38-45% carbohydrate which is comparatively high than cereal feed. Moreover, protein availability and digestibility are also high. Energy obtained from peanut haulms is around 2337 cal/kg of dry matter. The peanut plant also fixes atmospheric nitrogen for its growth and development and enhancement of soil fertility (Birthal et al., 2010).

1.3. Biological Nitrogen Fixation

Nitrogen is important for life, as it is a component of virtually all biomolecules. Although dinitrogen is enormously present in the atmosphere however N2 is not kinetically reactive toward either reduction or oxidation and metabolism of this elemental source is performed by most of the organisms. Therefore, an unstable form of nitrogen acquisition is essential for the growth, development, and existence of each organism. Luckily, some of the prokaryotic organisms could form ammonia from dinitrogen reduction and thus play the role to maintain nitrogen stable levels in the biosphere of earth. The nitrogenase enzyme system is the required biochemical machinery for the process of biological nitrogen fixation. Dinitrogen reduction to ammonia is not only catalyzed by nitrogenase, but it also catalyzes the reduction of several substrates like cyanide, azide, or acetylene and protons reduction to hydrogen (might be a mandatory portion of dinitrogen reduction). Nitrogenase catalyzed substrate reduction entails three basic types of electron-transfer reactions:

(1) Transfer of electron at the active site to the substrate within MoFe protein.

(2) The electron carries Fe protein reduction such as dithionite in vitro or flavodoxins and ferredoxins in vivo.

(3) In the MgATP-dependent process, transfer of single electrons to MoFe protein from Fe protein, with two hydrolyzed MgATP per transfer of electron.

The activity of nitrogenase is relatively slow, with a per electron turnover time of ∼5 s-1 (Howard and Rees 1996). An essential cycle of protein complex association and dissociation is involved in each electron-transfer step between MoFe and Fe protein the determine the overall rate of the reaction (Hageman and Burris 1978; Thorneley and Lowe 1983).

$$N2 + 8H+ + 8e- + 16MgATP = 2NH3 + H2 + 16MgADP + 16Pi$$

Annually, the worldwide biological nitrogen fixation process contributes to 175 and 100 million tonnes (Tg) N, reported by Burns and Hardy (1975) and Delwiche (1970) respectively (Burns and Hardy 2012; Delwiche 1970). Later, at the international conference in Sweden, the estimate was mentioned 122 Tg N by downgrading natural grasslands and forest fixed N. Annual fixation of 122 Tg N was supported by Burris and notified the value is compatible with values for the carbon cycle globally (Burris 1980). This estimation for nitrogen fixation includes both natural system and agriculture system and was derived using N balance and N difference methodologies, and derived acetylene reduction. The different symbioses and N2-fixing organisms found in terrestrial natural ecosystems and agricultural ecosystems are shown in Fig 1. Burris and Galloway estimated the annual global nitrogen fixation for cultivated land without tropical savannas are at 33 Tg (range 25-41 Tg) and 43 Tg (32-53 Tg) (Burris 1980; Galloway et al. 1995). Global nitrogen fixation estimates suggested by Galloway included all expects of N cycle, in cultivated agriculture systems is 32 Tg N/year (Galloway et al. 2004). Few estimates of symbiotic biological nitrogen fixation for tropical rain forests suggested ∼24% estimates of global natural terrestrial BNF annually (Cleveland et al. 1999). However, in the tropical forest, the richness of nitrogen fixation in legumes suggests that estimation of symbiotic biological nitrogen fixation is relatively high (Crews 1999).

Only blue-green algae and bacteria have evolved the ability to fix nitrogen from the atmosphere. Nitrogen fixation genes are involved in this process which is identified in particulars in five different bacterium *klebsiella pneumoniae* (Ausubel et al. 1977; Brill 1975; Cannon et al. 1977; Kennedy and Dixon 1977; Shanmugam et al. 1978; Shanmugam and Valentine 1975; Streicher et

al. 1971). Nitrogen fixation genes in Rhizobium spp formed root nodules in leguminous plants are derepressed (activation of nitrogenase activity in the presence of NH4+), leading to the production and transfer of fixed nitrogen to the outside environment (host cell chloroplast). Therefore, the de-repression of nitrogen fixation genes plays an essential role in the production of fixed nitrogen and the food chain of men on the global level (Shanmugam et al. 1978).

The biological nitrogen fixation process offers an economically attractive and environmentally friendly way to reduce external nitrogen input and improve the quality and quantity of crops. According to FAO estimates, it is suggested that 175 Mt of nitrogen are fixed annually worldwide by biological nitrogen fixation, materially contributing to a reduction in dependence on synthetic fertilizers and the sustainability of agriculture and agroforestry (Roy et al. 2006). Therefore, emphasis should be placed on developing new production methods that are sustainable both agroforestry and economically. About 55% of nitrogen required for growth and development is detained from biological nitrogen fixation in the peanut that could be enhanced (Hardarson 1993). Leguminous plants with low biological nitrogen fixation need more nitrogen as basal dose fertilizers for growth and development.

1.4. Biological Nitrogen Fixation in Legume

Legumes are generally used for fodder, fuel, food, timber, and shade. They are features of cropping system, Grazing system, the plantation system, and agroforestry system. In the cropping system legumes are grown intercropped with cereals or rotation. The grazing system includes vast grazing in semi-arid regions of natural vegetation and intensive type of agriculture. Legumes cover crops or give shade in plantation systems or where in the inter-row space of coffee, rubber, cocoa, and oil palm, shade trees are cultivated. In the agroforestry system, in combination with animals and crops, shrub legumes are cultivated for high sustainability and productivity of the farming system (Peoples and Craswell 1992).

Food legumes provide 90% of human dietary protein and 90% of the calories to tropical regions. Generally, consumption of legume seed provides 17-34% protein, percentage of dietary protein intake of legumes vegetables, and seed is 15-25%. Various legumes sources consumption in wide variation is due to animals' importance in the rural economy and due to many types of crops cultivated (Rachie and Roberts 1974; Wijeratne and Nelson 1987). Some species are grown as pulses (cowpea, common bean, black and green gram, chickpea, pigeonpea) or oilseeds (soybean

and groundnut), but some cops also have localized use for example rice bean, Kersting's groundnut, lablab bean, bambarra groundnut. Crop legume distribution is generally determined by their adaptation to the environment or climates (Rachie and Roberts 1974; Wood and Myers 1987). Nitrogen derived for individual species from biological nitrogen fixation range from 0.10- 0.88 (pigeonpea), 0-0.95 (soybean), 0.08- 0.89 (cowpea), 0.22-0.92 (groundnut), and 0.17-0.85 (chickpea). Nitrogen fixation for all these different legumes ranging from 0-450 kg N ha -1. Nitrogen fixation levels were dependent upon inoculation, n-fertilizer application, soil fertility, water supply, and crop rotation. Legumes might consume nitrogen fertilizer or nitrogen in the soil, where there was low nitrogen fixation. Nitrogen and phosphorous have strong inverse relation where there are low phosphorous, nitrogen is high in the soil, and results in low biological nitrogen fixation (Yoneyama et al. 1990). An important limitation of nitrogen fixation is the negative relationship between soil P and N available to plant. In intercropping system, the quantity of nitrogen fixation depends on species in the intercropping system, morphology, crop management, and density of legumes in the intercrop mixture. Competitive abilities for nitrogen available in the soil might stimulate biological nitrogen fixation. Legumes having climbing habits and indeterminate growth are commonly more successful.

1.5. Contribution to Livestock

Ruminant productivity can be improved by increasing both annual live weight gains and potential stocking rates. The relationship between leguminous material dietary intake or pasture sward legume content and animal performance has been found linear. Such retorts may be attributed to enhanced production of forage, quality of forage, and/or forage intake. Annual forage legume yield of nitrogen can be appreciable and considerable (e.g., potential of >700 kg Nha-lyr -1 in tree legumes) amounts of organic N that often amass in soil (Blair et al. 1990; Vallis 1972). The enhancement of soil nitrogen status by forage legume leads to a considerable increase in total herbage yields and an increase in the grass. Legume inclusion can add to a slow potential decline in plant productivity and animal carrying capacity which happens as pastures age (Haque and Jutzi 1984). Ultimately, the production of a ruminant is dependent upon the intake and digestibility of dry matter. In the rumen, rate, and extent of digestion, in turn, controlled the intake. Underfeeding conditions, the maintenance ratio should be the fodder of 1.15% N (7.8% crude protein) in dietary intake and beef cattle, to meet the requirement of protein 1.74% nitrogen is adequately considered. Nitrogen content in tropical grass species is often low (Little et al. 1989). Although to maintain an

optimum level of nitrogen in the grass with nitrogen fertilizer, however, the required applicability rates (e.g. >400 kg N ha -I yr -~) make it a naive approach. Instead, 2-4% nitrogen in the foliage of legumes is used for a high supplement of protein (Little et al. 1989). Legume proportion enhancement in the diet will increase digestibility and protein content and entire diet voluntary intake. Moreover, legume inclusion in the pasture can increase the nitrogen content of the grass associated (Bryan and Velásquez 1982).

1.6. Mechanism of Biological Nitrogen Fixation

Nodulation in legumes is an attractive example of effective symbiosis between plant and bacteria, portrayed by a precise balance between the microsymbiont and the host. Legumes have Nitrogen shortage however they have secondary metabolites like flavonoids which a signaling molecules to attract rhizobia and lead to the expression of nodulation genes in rhizobia (Abdel-Lateif et al. 2012). Node genes synthesis node factor (Nod F), that is lipo-chitooligosaccharide responsible for the induction of plant molecular response triggering root nodule formation (Oldroyd et al. 2011). NodFs bind with a class of receptor, receptor-like kinases (RLKs), which is an essential component of the host innate immune system and exploit the plant immune system for the formation of infectious thread in the root that facilitates rhizobial entry to the host plant (Tóth and Stacey 2015). Rhizobia enter the root epidermis legume using different modes such as infection thread (ITs) formation or plant-derived tubular structures and creak entry. Rhizobia uses ancient infection mode ‘crack entry’ for species of genus Arachis (counting Arachis Hypogaea) without the development of infection threads (Cai et al. 2013; Sprent and Platzmann 2001). IT traps rhizobia and keeps them isolated from the cytoplasm of the host cell.

Plant-rhizobia interaction was monitored in the early stages on the usage of live tissue images, aiming at the transferring of bacteria from the unframed environment to trapped IT (Fournier et al. 2015). In the formation of IT, the first globular apoplastic section is raised by an infection that holds bacteria. Later, the infection chamber is modified to the Its of typical tubular structure from compartmental radial morphology (Fournier et al. 2015). Its transfer rhizobia to active cortical cells lead to nodule development, resulting in a new root organ nodule. In nodule, bacteroid is produced from bacteria and supply ammonia (NH3) to the host plant and in return, the host plant provides carbohydrates to rhizobia. Terminal differentiation of bacteroid is achieved through genome endoreduplication, membrane permeabilization and cell enlargement (up to 10X as

compared to the free-living bacteria size) (Mergaert et al. 2006), and nodule-specific cysteine-rich (NCR), plant antimicrobial peptides, rule this entire process which also shows resemblance to defensin-type factor (Alunni and Gourion 2016; Gully et al. 2018).

Legumes generate determinate or indeterminate nodules, differing in development and morphological stages (Ohkama-Ohtsu et al. 2015). Nodulation metabolic cost is very high which is why its mechanism is tightly regulated. Signaling peptides play role in the systematic and local control of lateral root and nodule formation, considerably in the early stages of nodule development (Djordjevic et al. 2015). Respiratory burst oxidative homologs (RBOHs), NADPH oxidases, activity accumulate Reactive oxygen species (ROS) which accumulate in distinct signal transduction pathways of plants that regulate the symbiosis of N-fixing bacteria and legume (Arthikala et al. 2014). ROS level enhanced in the root tip of Pheseolus Vulgaris with the exposure of NodFs while it reduces in the roots of M. truncatula in the first hour of NodFs treatment which shows ROS association with the downregulation of MtRBOH3 and MtRBOH2 genes (Cárdenas and Quinto 2008; Lohar et al. 2007), while the use of NADPH oxidase inhibitor harms both early interaction of rhizobia in root hair and ROS production (Peleg-Grossman et al. 2007).

Molecular activities in rhizobial infection and nodule development are under the control of phytohormone. Ethylene negatively regulates symbiosis of legume and rhizobia, by silencing the NodFs activated signaling pathways at different stages (Guinel 2015). M. truncatula roots were inoculated with Sinorhizobium medicae to study phytohormone's role in the nodulation process with omics approaches (Larrainzar et al. 2015). Auxin's role in nodulation is not well defined, however, it might be possible that cell division in the infection process is associated with it (Breakspear et al. 2014; Schaller et al. 2015). In both nodule development and nodule primordia cytokinin signaling responses required cytokinins (Reid et al. 2016). Cytokinin oxidase and dehydrogenase genes are found to be regulated by NodF in the initial stage of nodule formation in Lotus japonicus. Another emerging player in symbiotic nodulation controlling is Jasmonate. In Astragalus, it was found that the ZIM-domain protein of jasmonate interacts with leghemoglobin (Li et al. 2015). It was suggested that jasmonate is required for bacteroid development, nodule number, and nitrogenase activity which showed the novelty of jasmonate in the symbiosis of legume-rhizobia.

In the host plant number of nodules is limited by the autoregulation of nodulation (AON), systematic signaling pathway, when rhizobia and host plant symbiosis takes place (Mortier et al. 2012). Recently studied in model legume M. truncatula, root signaling peptides are transferred to stem where they attached to shoot receptor complex and induce signaling pathway that harms nodule development. This mechanism needs the communication of CLV2 (CLAVATA) and CRN (CORYNE) proteins with SUNN receptors which are playing important role in the monitoring of root meristem activity (Crook et al. 2016).

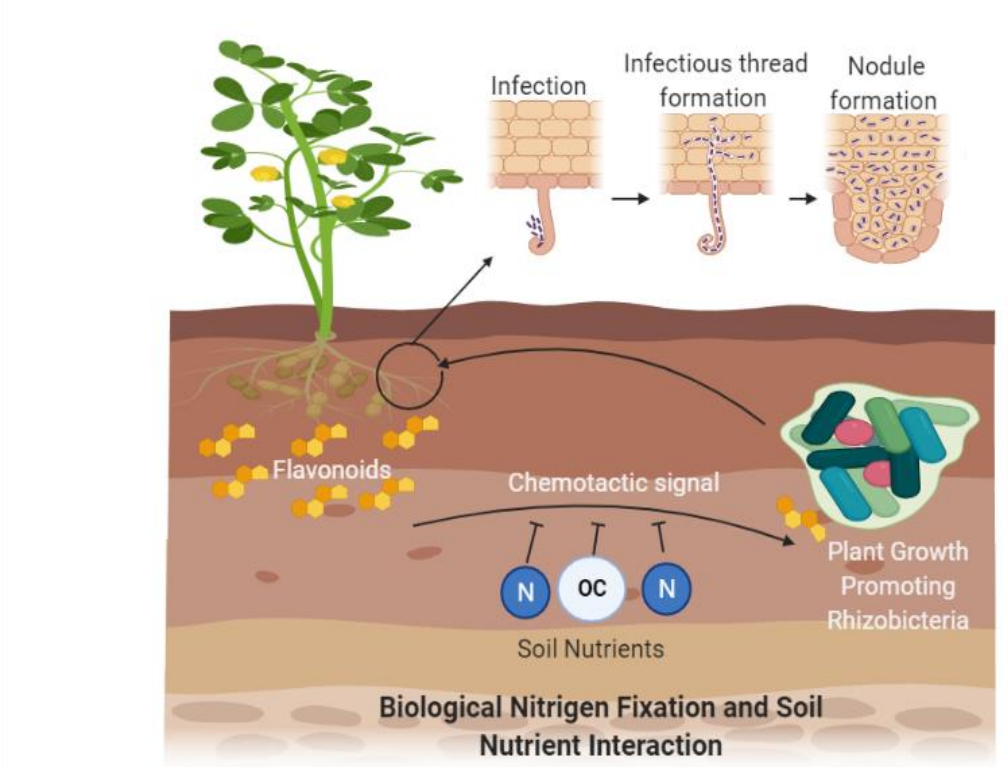

Fig: 2. Mechanism of biological nitrogen fixation and soil nutrient interaction with chemical signal.

1.7. Role of Flavonoids

Chemical signal molecules are essential for plant-microbes interaction, plant growth, and development (Cesco et al. 2012). Particularly, a low-molecular-mass compound flavonoid that exists in an extensive range of different structures can serve to deter or enhance plant yield by controlling plant-microbes communication (Cesco et al. 2012). It is central to plant-microbes symbiosis which increases the uptake of the nutrient, involving interaction between nitrogen-fixing bacteria and plant, as well as arbuscular mycorrhizal bacteria and host plants (Cesco et al. 2012).

Signals of flavonoids play an essential role in nourishing beneficial communities of the rhizosphere and might aid in the production of the symbiotic relationship of non-nitrogen-fixing crops (Mus et al. 2016). In response to pests, plants regulate the production of flavonoids as these compounds are the plant defense role in the roots against pathogens by showing antifungal and antimicrobial activity (Ahuja et al. 2012).

Manipulation of the transition of the chemical signal of flavonoids might be a helpful technique in pathogen management (Khan et al. 2006). Chemical information is encoded by flavonoids that are affecting all types of biological communication in the soil and hence regulate and underlie ecological processes on a large scale like nutrient cycling and community dynamic (Kessler and Kalske 2018). Plant fitness is affected by flavonoids expression; therefore, it is vital to study the environmental feedback and flavonoids fate according to their bioavailable concentrations. It is well studied that the loss of flavonoid signal is important for biodegradation, however, few studies have revealed the abiotic factors influence the chemical signal of flavonoids in soil (Shaw and Hooker 2008).

Soil properties have wide variation which influences numerous chemical availabilities including metal, pollutants, and nutrients (Kleber et al. 2007; Ren et al. 2018), showing physiochemical characteristics of soil might influence the flavonoids molecules bioavailability. One of the abiotic properties of soil is organic carbon (OC), which is principally essential to examined because plant growth rate and its delivery to the ecosystem influence the levels of OC in soil (Roth et al. 2019). Moreover, the strategies of soil management, consisting of the addition of organic amendments (e.g., compost, pyrolyzed OC, and wood) and plant litter, can highly affect the OC level in soil (Ogle et al. 2005). Typically, a high OC level is thought to enhance the symbiotic relationship and plant productivity, however, it is unclear whether the bioavailability of plant-microbes symbiotic signals is directly influenced by OC or not (Oldfield et al. 2018). The mechanism through which the soil matrix and OC could abiotically interfere with the biological signals, flavonoids, might be reactions with dissolved OC (DOC) and sorption into particulate OC (POC). These mechanisms might play an essential role in the modification of plant-microbes signal transmission (Gao et al. 2016). There are various flavonoids with distant functional groups and chemical properties, in the soil matrix these mechanisms might differently affect the movement of flavonoids. However, there

is no direct evidence available to show the interaction of DOC or POC with flavonoids altering plant-microbes communication.

The chemical structure of flavonoids also affects the half-life of signals in the soil equally, which is also unclear. Studies about the persistence of flavonoids reveal a wide range of half-life in the soil. Formononetin and naringenin have different biodegradation times as they have a different number of functional and conjugation, observed scales of day-long time (Shaw and Hooker 2008). Some studies suggest that flavonoid existence in the soil is related to seasons (Sosa et al. 2010). However, the signal loss mechanism has not been examined in these studies, which makes it difficult to produce a potential model of persistence of flavonoids. Chemical variation in the backbone of flavonoids has been linked with their biological activities (Kim et al. 2010). However, structural variation in flavonoids affecting the interaction with OC in the soil is not well studied. Decreased nodulation by DOC was examined as soil chemistry accounts for plant-microbes interaction (Del Valle et al. 2020).

Nodulation and biological nitrogen fixation are promoted by three main flavonoids (hesperetin, genistein, and naringenin), shown in different studies (Devos et al. 2006; Mapope and Dakora 2013; Mariotti et al. 2009; Novak et al. 2002). Classes of flavonoids are given in **table 1.** Nitrogen levels in soil and intercropping system influence the secretion of hesperitin, genistein, and naringenin from leguminous crops (Li et al. 2016; Liu et al. 2017). Intercropping system and Nitrogen supply of wheat and faba bean altered the amount and contents of flavonoids (Liu et al. 2020).

Table: 1. Main classes of flavonoids are summarized.

Classes	Flavonol	flavone	Flavonone	Xanthone	Isoflavone	Biflavone
1	Quercetin	Chrysin	Eriodictyol	gentisin	Formononetin	Amentoflavone
2	Kaempferol	Butin	Liquritigenin		Genistein	
3		Apigenin				
4		Luteonin				
5		Fisetin				

Secondary metabolites such as flavonoids have an important function in controlling the interaction between the environment and plants (Hauggaard-Nielsen et al. 2009). In the root, exudate flavonoids induce genes that are responsible for the process of nodulation in legumes by rhizobium. In pathways of general response for plant-plant, flavonoids are accountable for stimulating action and cascade signaling and in vitro after root exudate perception, are responsible for signal transduction (Bais et al. 2006; Murphy et al. 2000; Pourcel et al. 2007; Walker et al. 2003). However, the effect on flavonoid exudation, modulation process, and biological nitrogen fixation at a distinct level of nitrogen fertilizer is still undiscovered in the leguminous system. In the root exudate of faba bean six different flavonoids were found among them genistein, naringenin, and hesperetin had a significant correlation with the number of nodules and dry weight of nodules (Liu et al. 2019).

Nodulation involves symbiotic signaling which initiates two parallel developmental processes i.e., bacterial infection and organogenesis of the nodule (Oldroyd et al. 2011). Upon chemotactic signal (flavonoid) by plant, rhizobia produce signaling molecules know as nod factors (NFs) that signaled back to plant for bacterial entry into roots (Oldroyd et al. 2011). Rhizobia and host plant specificity are determined by NF which triggers the infection process and nodules formation (Kouchi et al. 2010). Identification and classification of the nodulation genes in Arachis hypogaea are useful for future research on the expression of these genes, as to date no study has been performed on genome-wide characterization and identification of nodulation genes in Arachis Hypogaea.

Former use several fertilizers and organic matter to fulfill the nitrogen requirement of the plant for growth and development. However, nitrogen sources and organic matter have a negative effect on biological nitrogen fixation. This research will evaluate the effects of different nitrogen sources, and organic carbon, provided in soil on several flavonoids containing a different number of hydroxyl groups, nodulation, and rate of nitrogen fixation in peanut plants individually. The result of our research will show us amended soil, giving high growth and development with maximum biological nitrogen fixation. Our research is a step toward optimum cropping strategy and might also help in reducing the use of nitrogen fertilizers which have harmful effects like leaching of nitrogen fertilizer, water, and soil contamination and low efficiency in utilization of nitrogen. The use of fertilizer/nitrogen sources and organic matter in farming have increased greatly which are

expensive. Our research will determine such a combination of nitrogen source and organic matter which will have maximum output in farming. In short, we are increasing the quality and decreasing the number of existing fertilizers and organic matter use in farming by studying their effects comparatively, which will be economical. Beneficial outcomes of the study might be less use of fertilizer, high plant growth, and development, maximum biological nitrogen fixation which is ecofriendly, economical for farmer and country, and low leaching of industrial fertilizer and contamination of soil. In the future might be this research leads to making soil composite favoring flavonoid biosynthesis, exudation from roots, and symbiotic signaling that might be applicable in every agricultural land where fertilizers and organic matter are in use for plant growth and development.

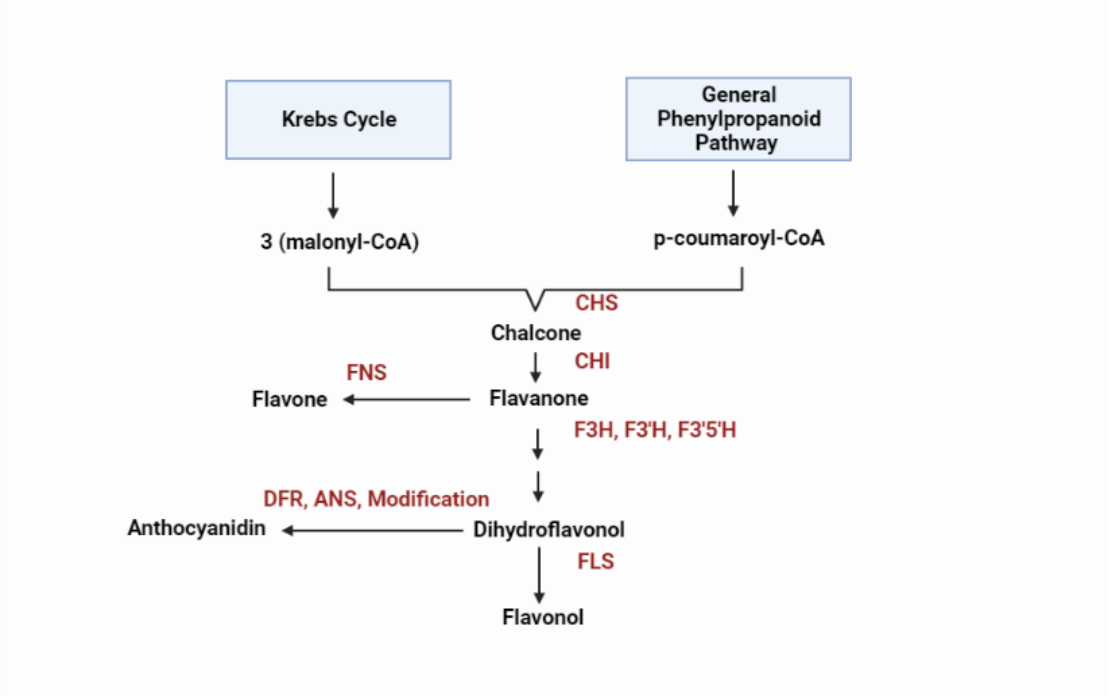

Fig: 3 Schematic view of flavonoids biosynthetic pathway is shown. It starts with the condensation of malonyl-CoA three molecules from Krebs cycle and p-coumaroyl-CoA from phenylpropanoid pathway. In red letters key enzymes are shown. Single biosynthetic step is shown by solid arrow. CHI, chalcone isomerase; CHS, chalcone synthase; F3′H, flavonoid 3′-hydroxylase; F3H, flavanone 3-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; ANS, anthocyanidin synthase; DFR, dihydroflavonol reductase; FNS, flavone synthase; FLS, flavonol synthase (Deng et al. 2018).

Aim and Objectives

1. The aim of the study is to evaluate the effect of organic amendment and nitrogen sources on the growth and development of *Arachis hypogaea* with the given objectives. Insilico identification and analysis of nodulation genes in peanut. To identify the effects of urea fertilizer with or without application of biochar on growth and physiology of *Arachis hypogaea.*

Chapter 2: Review of literature

The given literature review comprehends the work done on the role of flavonoids in nodulation and the effects of organic amendment and nitrogen sources on plant-microbes interaction and biological nitrogen fixation in various plants for plant growth and development.

Del Valle et al., (2020) investigated the influence of organic amendments and OC on signals of three different flavonoids (quercetin, luteolin, and naringenin) and their bioavailability (Del Valle et al. 2020). Flavonoids signals are repressed up to 70% with a higher amount of organic carbon. Repression magnitude depends on the chemical structure of flavonoid signaling molecules, sources of plant-derived organic carbon, and accessibility of metal ions. More repression occurs between flavonoids and dissolves organic carbon which is 63% rather than sorption of flavonoids to organic carbon particulate. The experiment showed that signaling interruption by organic carbon between nitrogen-fixing microbial symbiont and legume results in a 75% decrease in the formation of the nodule. The concentration of flavonoids measured via high-performance liquid chromatography was compared with that measured via microbial biosensors. These analyses evaluate that abiotic reaction and sorption are the main factors in signal loss. It was found that abiotic reactions that are responsible for the loss of flavonoid signals are due to DOC rather than POC, which showed novel polyphenolic chemicals of nearly equal masses with heterodimers flavonoids. DOC potentially decreases flavonoids signals having three and four hydroxyl groups naringenin and luteolin but not affecting the signal of flavonoids having five hydroxyl groups like quercetin. Nodulation is decreased by DOC, as soil chemistry accounts for plant-microbes interaction.

Liu et al., (2020) studied that nitrogen supply and intercropping system of faba bean and wheat altered the proportion and contents of flavonoids (Liu et al. 2020). An experiment performed with three N supply levels that are excessive, adequate, and deficient with three replicates in a complete block manner randomized. At low nitrogen levels (50%), intercropped wheat released more

flavonoids as compared to moncropped wheat at tilling and flowering stages. Naringenin releasing from the wheat root enhanced at all levels of nitrogen supply, however, hesperidin and genistein secretion enhanced at low nitrogen levels. At the flowering stage, the difference in the release of flavonoids is not substantial at the different nitrogen supply levels. To sum up, flavonoid secretion decreases with the increase of nitrogen supply. Flavonoids' greater accumulation on the faba bean root surface might increase the nodule dry mass and nodule number (Liu et al. 2017; Morris and Djordjevic 2006), which might enhance the rate of biological nitrogen fixation and hence yield of the crop (Liu et al. 2020).

Liu et al., (2019) explored that with the increased level of nitrogen fertilizer nodule dry weight, nodule number, and flavonoid exudation of faba bean decreased in the intercropping system with wheat (Liu et al. 2019). Six different types of flavonoids were seen in the root exudate of faba bean but three of them hesperetin, naringenin, and genistein shown a potential correlation with nodule dry weight and nodule number.

Peng et al., (2017) performed transcriptome analysis of Arachis Hypogaea L. showed differently expressed genes (DEGs) which include many orthologs to know genes of symbiotic signaling pathway such as ERN1, NSP2, and NFR5 (Peng et al. 2017). Recognition of NFs is controlled by NF receptor genes such as NFP and LYK3 in M. truncatula and NFR5 and NFR1 in L. japonicus (Arrighi et al. 2006; Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003). Moreover, DMI2 and receptor-like kinases SYMRK in M. truncatula and L. japonicus respectively, act as co-receptors in NF signaling (Oldroyd 2013). The recognition process of NF activates a secondary messenger that initiates nucleus calcium oscillations and responsible for proteins in nuclear membranes such as CASTOR, POLLUX, and nuclear pore (NENA, NUP133, and NUP85) (Oldroyd 2013). CYCLOPS and CCaMK complex decode calcium oscillations in the nucleus (Oldroyd 2013). NSP1 and NSP2, genes in the nodulation signaling pathway encode transcription factors, that further trigger transcription factors ERN and nodule inception proteins (NIN), symbiotic signaling pathway transmits these signals for the occurrence of nodule morphogenesis (Oldroyd 2013; Oldroyd et al. 2011). Furthermore, gene ontology enrichment analysis of orthologs reveals their contribution in metabolic process, oxidative-reduction process, catalytic process, defense system, and hormone biosynthesis (Peng et al. 2017). Therefore, identification and

characterization of nodulation genes would be fruitful for future research on the expression of these genes.

Zhang et al., (2016) performed numerous pot experiments using vermiculite to study the effects of P. liquidambari on the nodulation of peanut-bradyrhizobium. Results revealed that co-inoculation of P. liquidambari with bradyrhizobium enhanced the number of root nodules and accumulation of nitrogen in shoots by 28.25% and 29.71%, respectively. Nodule initiation and development of nodule were found to be accelerated. Activities of flavonoids synthesis enzymes potentially, secretion of quercetin, and luteolin which are rhizobia nod gene inducers in peanut, and formation of lateral root increased (Zhang et al. 2016)

Bustamante et al., (2020) evaluate the effect of distinct organic and inorganic fertilizer treatments and the enhancement of nutrient availability in soil on carbon-based secondary metabolites (flavonoids and phenolic compounds) and terpenoids in the leaves of Rosmarinus officinalis. Results reveal a positive correlation of the terpenoids' absolute amount with nitrogen and phosphorous content in leaves. However, the content of flavonoids and phenolic compounds was limited in the treatments of fertilization. Thus, a negative correlation between flavonoids and phenolic compounds was found with the nitrogen content in the leaves of Rosmarinus officinalis. The effect of fertilizer on the production of phenolic compounds and flavonoids is the direct tread-off of nitrogen between shikimic pathways which synthesized phenolic compounds and growth (Bustamante et al. 2020).

Etesami and Adl et al., (2020) reviewed that silicon in the soil increases growth, nodulation process, and consequently biological nitrogen fixation by several mechanisms under saline and non-saline conditions. Silicon in combination with non-rhizobia bacteria is beneficial for nodulation and biological nitrogen fixation in salinity stress (Etesami and Adl 2020).

Etesami and Adl et al., (2020) reviewed that silicon in the soil increases growth, nodule formation, and consequently biological nitrogen fixation by several mechanisms under saline and non-saline conditions. Silicon in combination with noLi et al., (2016) examined the intercropping of maize (Zea mays L.) with faba beans (Vicia faba L.), increased nodulation in Faba beans and overyielding is seen. Root exudates were responsible for the increase in the nodulation faba beans. The decline in the soil nitrate concentration by the intercropping of cereal is the reason for the promotion of nodulation in faba beans. The exudation of flavonoids is increased two-fold due to the

intercropping of maize. Maize root exudate when treated with the root of faba beans results in the enhancement in the chalcone-flavanone isomerase genes expression which is involved in the synthetic pathway of flavonoids that leads toward potentially increase expression of genes involved in the nodulation process. Maize exudate treatment in the roots of faba beans continuously reveals the up-regulation of nodulation genes like nodulin 93 ((ENOD93) and enhanced biological nitrogen (Li et al. 2016). n-rhizobia bacteria are beneficial for nodulation and biological nitrogen fixation in salinity stress (Etesami and Adl 2020).

Dakora and Phillips et al., (2002) reviewed that plant developmental processes are regulated by internal signals that rely on the nutrients supply from the soil to roots. Most mineral nutrients are taken through the rhizosphere by the plant where the interaction of the plant with micro-organisms stimulates the exudation from roots. Root exudate of the plant consists of a complex mixture of phytosiderophores, vitamins, nucleosides, organic acid anions, amino acids, inorganic ions such as HC03 -, OH-, H+, sugars, purines, gaseous molecules (C02, H2), root border cells, and enzymes which have an indirect and direct effect on the accumulation of mineral nutrients essential for plant growth. Aldonic acid and Phenolic such as flavonoids exudate from the root and significantly serve as main signals to attract rhizobia from biological nitrogen fixation. In a low nutrient, environment plants secrete root exudates as symbiotic signals for soil microbes that contribute to the procurement of nutrients. Nitrate availability in soil for plant growth as fertilizer compiles plants to release anions including hydroxyl ions in excess amount to maintain electric neutrality. Legume which has the capability of nitrogen reduction in the nodules present in roots secretes protons in excess amounts. These protons lower the PH of the rhizosphere and decrease mineral nutrient availability as well as harm some of the soil bacteria functioning, such as rhizobia. Therefore, a naturally acidic environment challenges nutrient acquisition by the roots of the plant, and the root and survival of many beneficial microbes are threatening (Dakora and Phillips 2002).

Chapter 3: material and methods

3.1. *In silico* Analysis

In silico analyses was carried out for identified genes.

3.1.1. Identification and Annotation of Nodulation Genes in Arachis Hypogaea

Nodulation genes (NFP, SYMRK, NUP85, NUP133, CCaMK, CYCLOPS, NSP1, NIN, ERN1, Nod, Nol) of Lotus Japonicus and Arachis Guridansis were initially obtained from(R). Homologous FASTA sequences were retrieved upon searching these genes in NCBI (https://www.ncbi.nlm.nih.gov/). FASTA sequences were used as query to carry out Blastn searches in peanut genomic database Peanut Base (https://www.peanutbase.org/) and Peanut DB (http://bioinfolab.muohio.edu/txid3818v1/) with cutoff e-value of 0e. Peanut base is main database for peanut as contains genomic and genetic information for cultivars and wild relative of peanut. Its stores genes, genome sequence, and predictive function, the markers, genetic maps, germplasm map of origin, and germplasm resource linkage is also deposited in Peanut base (Dash et al., 2016). Arachis hypogaea and its cultivar transcriptome data is available on peanut DB web portal (Duan et al., 2012). To verify the reliability of results, all gene sequences were checked in the pfam database (http://pfam.xfam.org/) for domains and manually identified full-length genes, half-length genes and genes having no domains in ORF. We proceeded with 42 full-length genes after discarding redundant sequences with different identification number and same chromosome locus. To confirm the identity of the nodulation gene functions of domains present in ORF of these sequences were analyzed for nodulation process. The ExPASY translate tool (http://www.expasy.ch/tools/dna.html) was used to deduce the amino acid or open reading frames of *Nod* inducing genes. Then chemical and bio-physical parameters of *Nod* inducing 42 genes in *Arachis hypogaea* were predicted by protPARAM (http://expasy.org/tools/protparam.html) available at ExPASY by using

primary sequences of genes. These properties were predicted to explore important characteristics of genes e.g., protein length (aa), coding sequence (CDS), gene length (bp), molecular weight (MW), grand average of hydropathicity (GRAVY), isoelectric point (pI), instability index and aliphatic index (AI).

3.1.2. Multiple Sequence Alignment and Phylogenetic Analysis

The sequences of *Nod* inducing genes were retrieved from peanut base (https://www.peanutbase.org) and multiple sequences of full gene length were aligned by using MEGA 10.2.4 tool (https://www.megasoftware.net/) at default setting by using "align by muscle" and an unrooted tree was also constructed by using MEGA 10.2.4 tool with neighbor joining (NJ) algorithm. The bootstrap replicates of 1000 with 50% cutoff values were used to test the reliability of tree and then the tree was visualized. Phylogenetic tree is a diagram with several branches that explain the evolutionary relationship among biological species.

3.1.3. Gene Structure and Conserved Motif Distribution Analysis

Online gene display server (http://gsds.gao-lab.org) was used to predict Nod inducing genes structural information such as exon/intron patterns (Guo et al. 2007). BED file format for coding regions of the genes were used for gene structure prediction. Prediction for conserved protein prediction and significant function was determined by the MEME program (https://meme-suite.org/meme/). Some parameters were adjusted in this analysis such as: 3 residues, 25 distribution of motifs, optimum motif width, and any repetition number (Bailey et al. 2015).

3.1.4. Chromosomal Distribution and Gene Duplication Events

Peanut Base (https://www.peanutbase.org) was used to determine the chromosomal length and information about the position of NIG gene family. In twenty chromosomes of Arachis Hypogaea, distribution of NIG family genes and relative distances were visualized with online visualization tool PhenoGram (http://visualization.ritchielab.org/phenograms/plot). TB-tool software (https://github.com/CJ- Chen/TBtools) was used to evaluate segmental and tandem duplication events, selective pressure, and divergence time of genes, the non-synonymous

(Ka) and the synonymous (Ks) values (Chen et al. 2020). T=Ks/2x*MYA formula was applied for calculation of time divergence of genes, where x=6.56 x 10^{-9} and MYA= 10^{-6} (He et al. 2016). NIG gene family segmental duplication details was also illustrated in the diagram.

3.1.5. Subcellular Localization Prediction and Sequence Logos Analysis

The location of protein inside the cell helps determine the role of protein and its interaction with several other molecules and proteins. Prediction and understanding the functions of the NIG family proteins on the base subcellular localization was started with the translation of all genes into protein sequences with the help of online Expassy translate tool (https://web.expasy.org/translate/). Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/), an online website was utilized to trace the location of proteins (Chou and Shen 2010). Different locations predicted by Plant-mPLoc predictor for plant proteins are twelve such as Cell wall, Cytoplasm, Mitochondria, Nucleus, Peroxisome, Plasma membrane, Chloroplast, Endoplasmic reticulum, Extracellular, Golgi apparatus, Plastid, and Vacuole. Moreover, conserved, or non-conserved amino acid are determined with Sequence log in all genes. First, CLUSTALW (https://www.genome.jp/tools-bin/clustalw) was used to aligned all proteins of NIG family (Thompson et al. 1997) and then with WebLogo (https://weblogo.berkeley.edu/logo.cgi) sequence logos were generated (Crooks et al. 2004).

3.2. Wet Lab Analysis

Wet lab analyses were caried out to find the effect of urea fertilizer on biological nitrogen fixation.

3.2.1 Seed and Soil Collection

Healthy and pathogen-free seeds of peanut varieties (Golden) were collected from the department of oil, seed, and research at National Agriculture Research Centre (NARC), Islamabad, Pakistan. Sandy loamy soil from the peanut field was collected from BARI (Barrani Agriculture Research Institute), Chakwal.

3.2.2. Seed Germination

Seeds were de-husked and surface sterilized in 70% ethanol for 2 min followed by washing through autoclaved distilled water. Seeds are then spread on filter paper in a surface-sterilized safety cabinet to evaporate maximum ethanol. Completely dried seeds were then wrapped in aluminum foil and placed at 4oC for 48 hours for breaking seed dormancy. Seeds were then transferred to beakers containing moistened cotton. 4 to 5 seeds were placed in each layer of the cotton in a beaker and covered with aluminum foil to avoid penetration of lights. Sterilized forceps were used to transfer seeds to avoid contamination. The beakers were placed in dark cabinets at the temperature of 25-28oC. After ten days' seeds started germinating and were ready to transfer to soil. Seeds with equal germination rate were transferred into the pot for growth in the greenhouse.

3.2.3. Soil Analysis

Soil analysis was conducted at Barrani agriculture research institute (BARI), chakwal. Soil texture is Sandy loam, PH is 7.78, Saturation percentage (SP) is 32, ECe is 0.53 ds/m and organic matter is 0.49%. amount of K, P, and N is 121mg/kg, 6.2 mg/kg, and 0.025% respectively (Estefan et al. 2013).

3.2.4. Soil Preparation

The soil used for growing plants was prepared for eight treatments. The first four treatments contain soil media while the last four treatments have 1.5% biochar. The nitrogen sources were Calcium Nitrate tetrahydrate (Ca (NO3)2.4H2O), Ammonium sulfate (NH4SO4), and Urea (CH4N2O). All treatments are illustrated in given **tables 2**. Along with nitrogen sources, other soil nutrients such as calcium (Ca), Sulfur (S), potassium (K), phosphorus (P) were also added as a solution to all treatment as required per kg which is 15mg, 24mg, 18.6mg, 17mg, and 14mg, respectively. Soil nutrients requirements for soil are given in **table 3** below and their calculation is given in **table 4**. There were two batches and each batch consist of four replicates and soil per pot was 1kg.10ml solutions per pot were prepared for the nitrogen sources and soil nutrients and added to required treatments. For each treatment after adding solutions soil was mixed in a tub and after mixing one kg soil was added to labeled pots. Seeds were placed one inch

deep in the soil with roots in the downward direction. The seeds were then covered with soil. The temperature for growth was kept at 28oC with 16h/8h light condition.

Table 2: All treatments are shown in given table

Treatments	**Without Biochar**	**Treatments**	**With Biochar (1.5%)**
T 1	No Nitrogen (control)	T 5	No nitrogen (control)
T 2	Ca(NO3)2.4H2O	T 6	Ca(NO3)2.4H2O
T 3	NH4SO4	T 7	NH4SO4
T 4	Urea	T 8	Urea

.

Table 3: Soil nutrients requirements are shown in the table.

Required nutrients	Per hectare	Per Kg	Per 3Kg	Per 6Kg
Nitrogen (N)	30 kg	15mg	45mg	90mg
Calcium (Ca)	48kg	24mg	72mg	144mg
Sulfur (S)	37.2kg	18.6mg	55.8mg	111.6mg
Potassium (K)	34kg	17mg	51mg	102mg
Phosphorus (P)	28kg	14mg	42mg	84mg

3.2.5. Water Holding Capacity

For calculating the water holding capacity of the soil media, we took 3 pots for simple soil and 3 pots for soil containing 1.5% biochar. We did 5 holes in each pot in the bottom of the pots and Put tissue inside each of them. Then soil and soil having 1.5% biochar were added into the pots having empty space of one inch above in all pots. Then three pots having only soil were placed in one water tube and the other three pots having 1.5%

biochar in the other water tub. water was added to both tubs up to the level of soil in the pots. After 8 hours all pots were placed on inverted sieves for water drainage for an equal amount of time. The weight of the pots was measured which is W1. Then soil was removed from the pots covered in aluminum foil and dried in a drying oven for 4 to 5 hours at 105 C. soil weight was measured after drying (Wa). Pots were dried at room temperature weight was measured (Wb). We got values for W2 which is W2= Wa +Wb and water holding capacity was measured according to the given formula (Wang et al. 2014).

100 % Water holding capacity = [W1-W2) /W2] * 100

100% field capacity was obtained then 65% field capacity was measured as shown in given **table 5**. After sowing of seed, we added 65% water holding field capacity to all pots. Then measured the water loss on an alternative day. And maintained the water loss throughout the experiment as shown in the table. **Table 6 and 7** depicts water added to plants in batch one and batch two.

Table 5: Measurement of water holding capacity is shown.

	W1	Wa	Wb	W2 = Wa + Wb	WHC= [W1-W2)/W2] x100	100% WHC in 1000g soil	70% of total WHC	Average	Water added
Soil media 1	1098 g	820 g	47 g	867 g	26%	260ml	169ml	180.505ml	200ml
Soil media 2	1107 g	809 g	44 g	853 g	29.54%	295.4ml	192.01ml		
Soil + 1.5% BC 1	1147 g	777 g	48 g	825 g	39%	390ml	253.5	227.175ml	250ml
Soil + 1.5% BC 2	1155 g	830 g	52 g	882 g	30.9%	309ml	200.85		

3.2.6. Phenotypic Analysis

The number of leaves and branches were counted on 14th, 21st, 28th, 35th, 42nd, and 45th days after sowing. Shoot heigh was measured with meter rod on 14th, 21st, 28th, 35th, 42nd, and 45th day (Husain et al. 1990). Taproot length was measured on the 45th day (harvesting day). The number of nodules was counted for each plant after harvesting (Aina et al. 2019; Liu et al. 2019; Zhang et al. 2016). The fresh shoots and roots were

washed with deionized water, air-dried to remove the water adhering to them, dried at 70 °C for 72 h, and then their dry weight was determined (Luo et al. 2017). Dry weight is the biomass.

3.2.7. Chlorophyl Content

Chlorophyll A and B in plant tissue were determined by using a spectrometer. Leaf sample was taken directly after harvesting about 1cm square and 0.07mg and grinded with mortar and pestle. During grounding small amount of acetone and magnesium carbonate were added. The samples were collected in a small falcon tube and centrifuged for 2 minutes at 13000 rpm. All samples being analyzed will be read at 630, 645, and 750 nm wavelength (chlorophyll A = 630, and chlorophyll B = 645). Acetone was used as a control. Calculations for chlorophyll A and B and total concentrations were made after the absorbances are read all wavelengths. (Guo et al. 2017; Zhu 1993).

Formula for chlorophyll concentration: (11.6 × 665nm) – (.14 × 630nm).

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Chapter 4: Results

4.1. Insilico Results

4.1.1. Identification and Annotation of NIG Family in Arachis Hypogaea

In Arachis hypogaea, genome-wide studies of nodulation genes were performed previously by searching available data in public databases which highlight many genes playing role in the nodulation process. Total 15 NIG genes were identified on the base of sequence identity in Arachis hypogaea after removing redundant sequences with the same percentage and insignificant e-value. Blastn searches and gene annotations in peanutBase database were used to obtain NIG family exhaustive list with a cutoff e-value of 0e. To conclude open reading frames or amino acids for Nod inducing genes, the ExPASY translate tool was utilized. Nodulation features displayed for NIG proteins based on Pfam analysis. In table 1, names assigned to genes, their accession numbers, coding sequence length, and protein characteristics were shown. proPARM accessible at ExPASY was used to predict the essential biophysical and chemical characteristics such as protein length (aa), coding sequence (CDS), isoelectric point (pI), aliphatic index (AI), grand average of hydropathicity (GRAVY), molecular weight (MW), extinction coefficients (EC) by assuming all pairs of Cys residues form cystines, and their estimated half-life.

Nod inducing genes full-length coding sequence range from 543 bp (AhNKLM1) to 2958 bp (AhNPR4) and residues of the amino acid (aa) in their putative proteins comprised between 559 and 986 amino acids (aa), with as average of ~771 aa. 4.82 (AhNNLC1, AhNNLC2) to 6.73 (AhNKLM2) and 62975.18 (AhNKEF4) to 109443.61 (AhNPR4) are the range for theoretical pI and molecular weight. In genome-wide studies, Potential variation is detected in extinction coefficients, GRAVY inferring a high degree of complexity, and aliphatic index and among NIG gene family members of Arachis hypogaea functional diversification was also seen as shown in **table 8**.

Table 8: Protein length (aa), coding sequence (CDS), isoelectric point (pI), aliphatic index (AI), grand average of hydropathicity (GRAVY), molecular weight (MW), extinction coefficients (EC).

Sr. No	Gene ID	Chromosomes	Accession numbers	Nucleotide CDS (bp)	Length (aa)	PI	Mw	II	AI	EC	GRAVY	Half life
1	AhNMTL1	13	MZ169526	2772	923	5.71	103395.37	42.89	91.86	107120	-0.228	>10 hours
2	AhNMTL2	03	MZ169527	2772	923	5.76	103503.55	41.53	91.33	110100	-0.235	>10 hours
3	AhNKEF1	05	MZ169528	1703	567	5.83	63918.94	34.12	82.70	58370	-0.403	>10 hours
4	AhNKEF2	15	MZ169529	1702	567	5.91	63952.92	35.14	82.36	59860	-0.422	>10 hours
5	AhNKEF3	17	MZ169530	1682	560	5.58	63090.23	44.69	81.86	49320	-0.477	>10 hours
6	AhNKEF4	13	MZ169531	734	559	5.58	62975.18	45.54	82.70	49320	-0.458	>10 hours
7	AhNNLC1	16	MZ169532	2208	735	4.82	72061.97	56.54	51.93	24980	-0.065	>10 hours
8	AhNNLC2	06	MZ169533	2211	736	4.82	72096.97	56.12	52.00	24980	-0.061	>10 hours
9	AhNPR1	17	MZ169534	2877	958	5.60	106770.63	53.07	72.35	97885	-0.558	>10 hours

10	AhNPR2	08	MZ169535	2895	964	5.63	107484.54	53.70	72.20	99375	-0.563	>10 hours
11	AhNPR3	15	MZ169536	2952	984	5.42	109253.43	58.20	76.19	86550	-0.420	>10 hours
12	AhNPR4	05	MZ169537	2958	986	5.48	109443.61	57.56	75.14	86550	-0.438	>10 hours
13	AhNKLM1	11	MZ169538	543	762	6.60	84511.47	41.55	88.39	106980	-0.183	>10 hours
14	AhNKLM2	01	MZ169539	1887	628	6.73	69666.50	43.30	86.43	87040	-0.211	>10 hours
15	AhNNup1	16	MZ170086	2151	716	6.36	81199.26	51.55	98.35	150395	-0.214	>10 hours

4.1.2. Evolutionary Analysis of NIG Gene Family

Evolutionary relationship among for all identified 15 NOD (Nod Inducing Gene- family) inducing genes was studied. First sequences were aligned, and then a phylogenetic tree was created by utilizing the neighbor-joining method with 1000 bootstrap replicates. Evolutionary analysis distributed all genes of NIG family into three sub-families stated as NIG-a, NIG-b, and NIG-c as shown in **fig 4**.

NIG-a has two groups, NIG-a1 and NIG-a2 which contained nod melactin-tyrosine-LLR and nod kinase-EF hand conserved domains, respectively. Nod Melactin-tyrosine-LLR is involved in binding on receptor kinase, signal transduction, transfer of phosphate group, and protein-protein interaction. Nod kinase-EF hand genes are involved in signal transduction, transfer of phosphate group, and binding of calcium ions and structural domain of the signaling protein calmodulin.

NIG-a1 consist of AhNMTL1 and AhNMTL2 genes, and NIG-a2 comprised of AhNKEF1, AhNKEF2, AhNKEF3, and AhNKEF4 genes.

NIG-b has three groups, NIG-b1, NIG-b2, and NIG-b3 which contained conserved domains such as Nod Nucleoporin1, Nod Kinase-LysM, and Nod PB1-RWP RK, respectively. Nod Nucleoporin1 is responsible for the building blocks of the nuclear pore complex. Nod Kinase-LysM performs functions of signal transduction, transfer of phosphate group, and receptor for Nod factor. Functions as a protein-binding module through PB1-mediated heterodimerization or homo-oligomerization and nitrogen-controlled development are due to Nod PB1-RWP RK genes. NIG-b1 has AhNNup1 gene, NIG-b2 has AhNKLM1 and AhNKLM2 genes, and NIG-b3 has AhNPR1, AhNPR2, AhNPR3, and AhNPR4 genes.

NIG-c sub-family has only one group NIG-c1 which contained Nod Nsp1-like C-terminal on the base of conserved domain analysis, responsible for being involved in binding nuclear pore protein (Nup82). NIG-c1 consists of two genes namely AhNNLC1 and AhNNLC1.

The groups in the sub-families on the phylogenetic tree indicate that genes are paralogs in NIG family genes which revealed their evolutionary relationships. According to their functions on the base of conserved, all genes are categorized as rhizobial infection and nodule formation genes which leads to nitrogen fixation in the nodules.

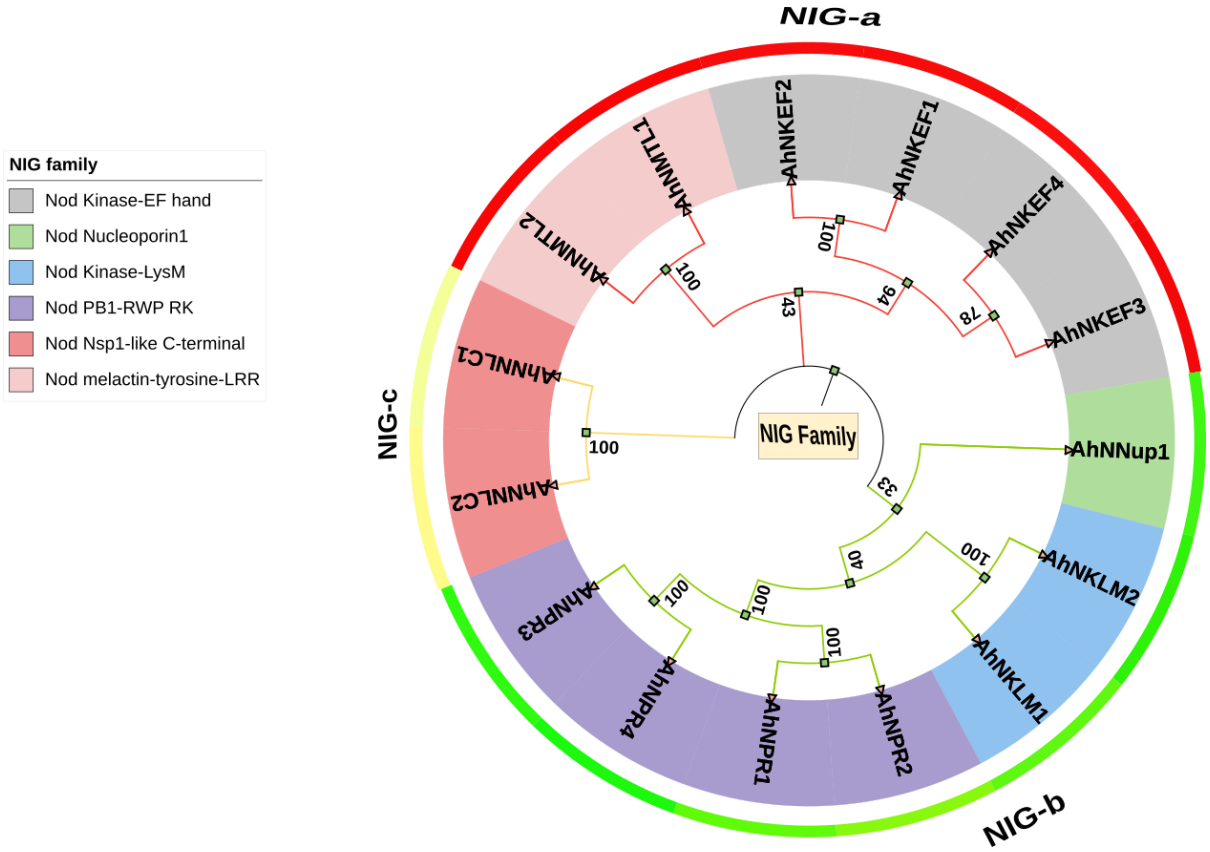


Fig 4. Phylogenetic analysis of NIG family genes.

4.1.3. Conserved Motif Analysis and Intron/Exon Organization of *NIG* Gene Family

NIG family genes conserved motifs were anticipated by employing an online MEME tool which is essential for understanding structural characterization and diversification of genes. Therefore, detailed information of all genes in NIG family including name, width, e-value, sites, and significant matches were demonstrated. Several motifs were ten with different amino acids, where the length of amino acid ranges from 21-41. Identified motifs, the sequence for each conserved motif, and their schematic distribution in all genes in sub-groups of NIG family are represented in **Fig 6.** Motifs numbers are comparatively greater in sub-family NIG-a than sub-family NIG-b. The numbers of motifs were comparatively more in NIG-a sub-family than NIG-b, comparatively and NIG-c sub-families has the lowest number of motifs which is two. The analysis suggests that Motif 8 (present at was found on 14 different sites, having 21 amino acids) in all three sub-families NIG-

a, NIG-b, and NIG-c expect one gene, AhNNup1 of sub-family NIG-b, signifying that it could be an important motif having conserved function related with NIG family genes and biological nitrogen fixation as shown in **table 9**. Motifs 1, 3, 5, 6, 8, and 9 were common in NIG-a sub-family, motif 3 and motifs 8 were common in NIG-b and NIG-c sub-family, respectively. Moreover, it is found that conserved domains are positioned at the same sites which suggest that majority of genes in NIG family are closely linked. The common composition of motifs represents that genes' structure might be highly conserved. Functional characterization and structural diversity of NIG family genes are vital to understanding the evolutionary history of this family.

For intron-exon structure analysis, BED files of NIG family genes were utilized, results indicated that all genes are full-length genes which means domain function in nodulation is inside CDS regions. However, the intron region lies upstream in the CDS region as shown in **Fig 5.** These analyses suggest that NIG family has maximum common structural conservation.

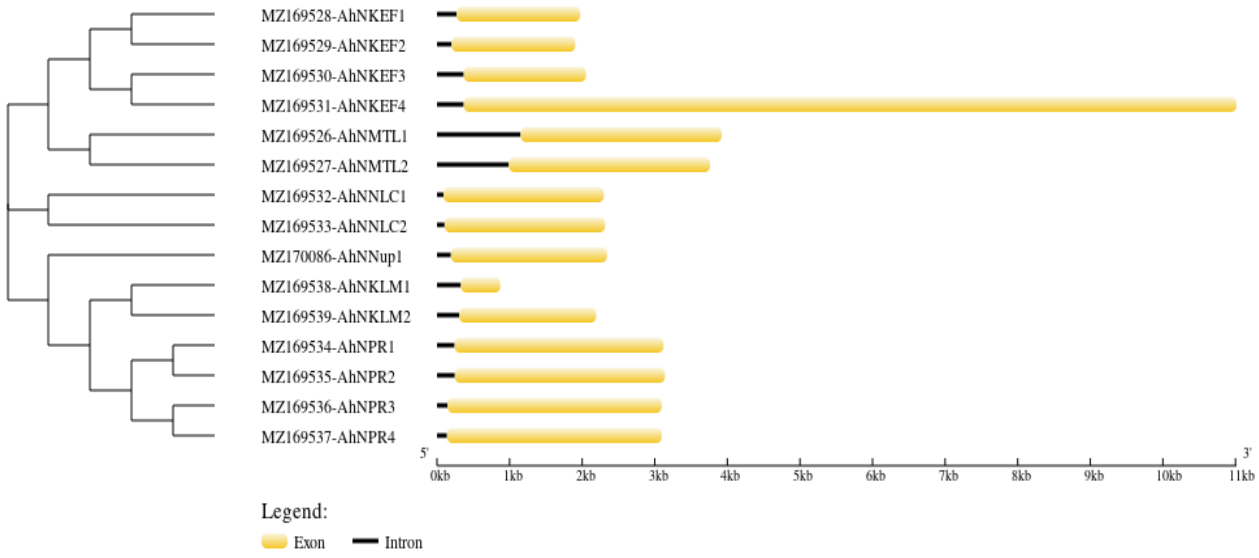


Fig 5. Intron-exon structural illustration.

Fig 6. Motif distribution illustration.

Table: 9. The MEME motif sequences and lengths in NIG family

Motif	Conserved amino acid sequences	e-values	Sites	Width
1.	RVYSPKVDVWSPGVILYELLSGREPFHAZ	3.0e-089	12	29
2.	RHFAGSLKDAAKNJGVCPTTLKRICRQHGIKRWPSRKIKKV	4.7e-073	4	41
3.	HLCHRQGVMHRDLKSENILLD	7.6e-053	11	21
4.	YEDPLNVHIVMELCEGGELFDRIIQRGHY	2.0e-052	4	29
5.	LGRKLGRGEFDIKYLDDDNET	6.5e-045	10	21
6.	DYGEFIAVTIHLNKLERDEHLREAFRYFD	1.6e-045	6	29
7.	SKTDYPLVHHARMFGLRAAFAIRLRSIHTGSDDYVLEFFLP	2.6e-057	4	41
8.	EKLTTRPEVEDVRRELEAMNL	7.4e-045	14	21
9.	GPGAVFTEIVGSPGYIAPEYL	2.4e-042	8	21
10.	HVPLEDIIREVDTDNDGRIDYGEFAAMMK	1.3e-048	6	29

4.1.4. Chromosomal Location and Gene Duplication of *NIG* Genes

Chromosomal location was investigated on all 20 chromosomes of Arachis hypogaea for all 15 genes. All genes were distributed widely throughout the genome of Arachis hypogaea. Genes were named and mapped on their respective chromosome according to their order as shown in **figure 7**. However, the distribution of genes on chromosomes showed uneven distribution which indicated genetic variation due to the evolutionary process.

NIG family genes were found on Chr1, 3, 5, 6, 8,11, 13, 15, 16, and 17 while there were no genes on Chr2, 4, 7, 9,10, 12, 14, 18, 19, and 20. Among 20 chromosomes, 5 chromosomes harbored only one number of genes (Chr1: AhNKLM1, Chr3: AhNMTL2, Chr6: AhNNLC2, Chr8: AhNPR2, and Chr11: AhNKLM1). Chr5, Chr13, Chr15, Chr16 and Chr17 have high number of genes i.e., two genes (AhNKEF1/ AhNPR4, AhNKEF4/ AhNMTL1, AhNKEF2/ AhNPR3, AhNNup1/ AhNNLC1 and AhNKEF3/ AhNPR1, respectively) as compared to others.

During gene family expansion duplication mechanisms occur in plants which involves tandem duplication, segmental duplication, and whole-genome duplication. Therefore, to better understand the relationship among NIG family genes and duplication events within the genome of Arachis hypogaea, we analyzed the mechanisms of duplication during the evolution of NIG family genes as illustrated in table 2. Two pairs of duplicated genes were identified by analyzing the similarities and sequence coverage of 15 NIG genes such as AhNMTL1- AhNMTL2 and AhNNLC1- AhNNLC2, resulted from segmental duplication event as shown in fig 4. There was no whole-genome and tandem duplication event which suggested that during the expansion of NIG family in Arachis hypogaea segmental duplication contributed largely. By using the KaKs calculator, Ka and Ks values were calculated and the obtained information was utilized to define the selective evolutionary pressure. Both gene pairs have Ka/Ks<1 which showed purification selection. Moreover, segmental duplication of these pairs of genes was projected between 0.28674973 and 0.46897178 million years ago as shown in **table 10**.

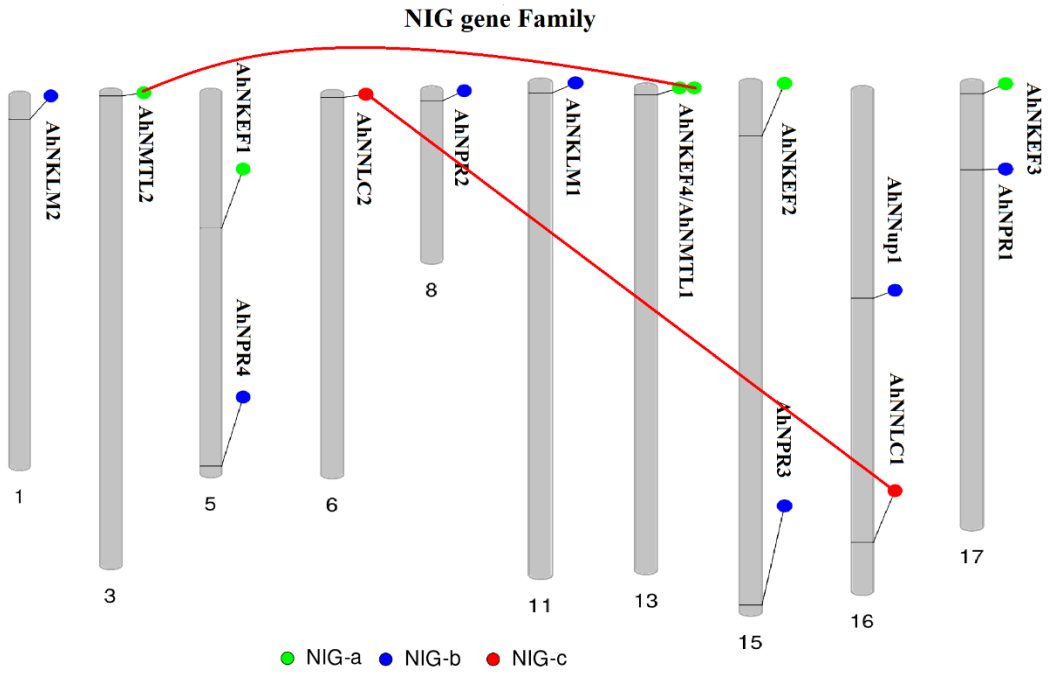


Fig: 7. Chromosomal distribution of all 15 genes.

Table: 10. Ka, Ks, and Ka_Ks calculation and divergent time of duplicated genes in NIG family.

S.No:	Paralogous pairs	Ka	Ks	Ka_Ks	Duplication	Time (Mya*)
1	AhNMTL1-AhNMTL2	0.00615291	0.01876564	0.32788171	SD	0.46897178
2	AhNNLC1-AhNNLC2	0.00376216	0.03565028	0.10552952	SD	0.28674973

4.1.5. *NIG* Family Protein Subcellular Localization and Sequence Logos Analysis

Protein localization is achieved with the help of Plant-mPLoc analysis as shown in **figure 8.** It was predicted that among all proteins of NIG family, 12 proteins are sited at a single position. Single positions for their location are either cell membrane or nucleus or chloroplast. Two proteins are located on two locations i.e., cell membrane and nucleus, and one protein lies on all three locations

cell membrane, nucleus, and chloroplast as shown in fig. The pattern of protein conservation for all 15 genes is discovered and evaluated with the help of Sequence logos. Sequence logos were generated of all aligned amino acid residues of NIG family genes to find whether, in all 15 genes, NIG family proteins are conserved throughout evolution as shown in fig. Analysis reveals that conservation of protein sequences ranges from moderate to a high level at several positions from N to C terminal as shown in **figure 9**.

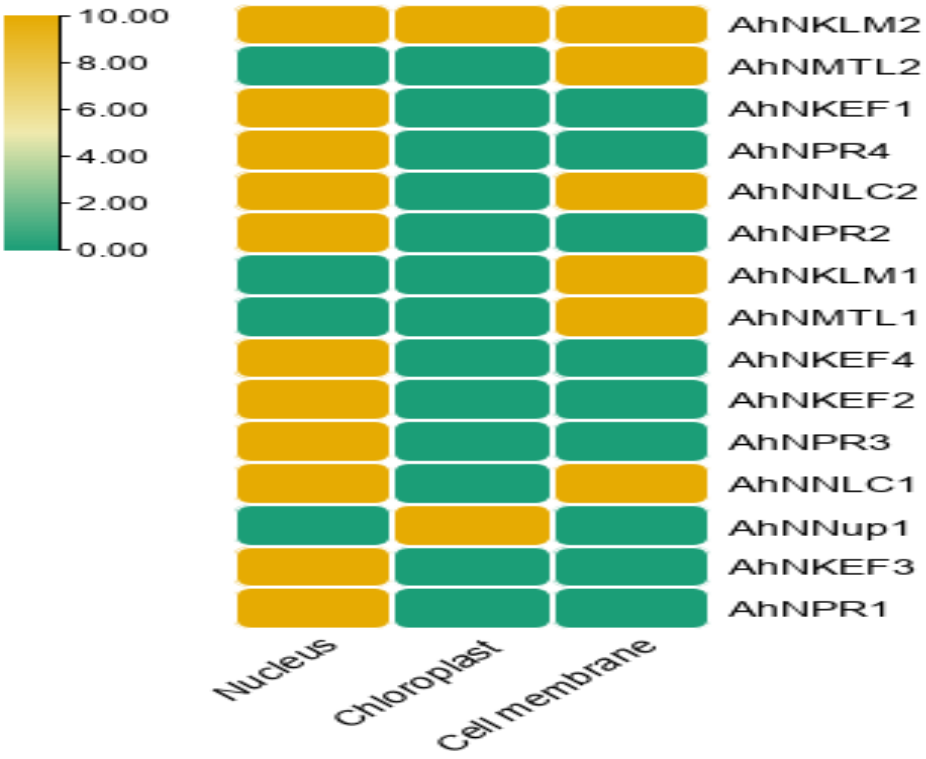
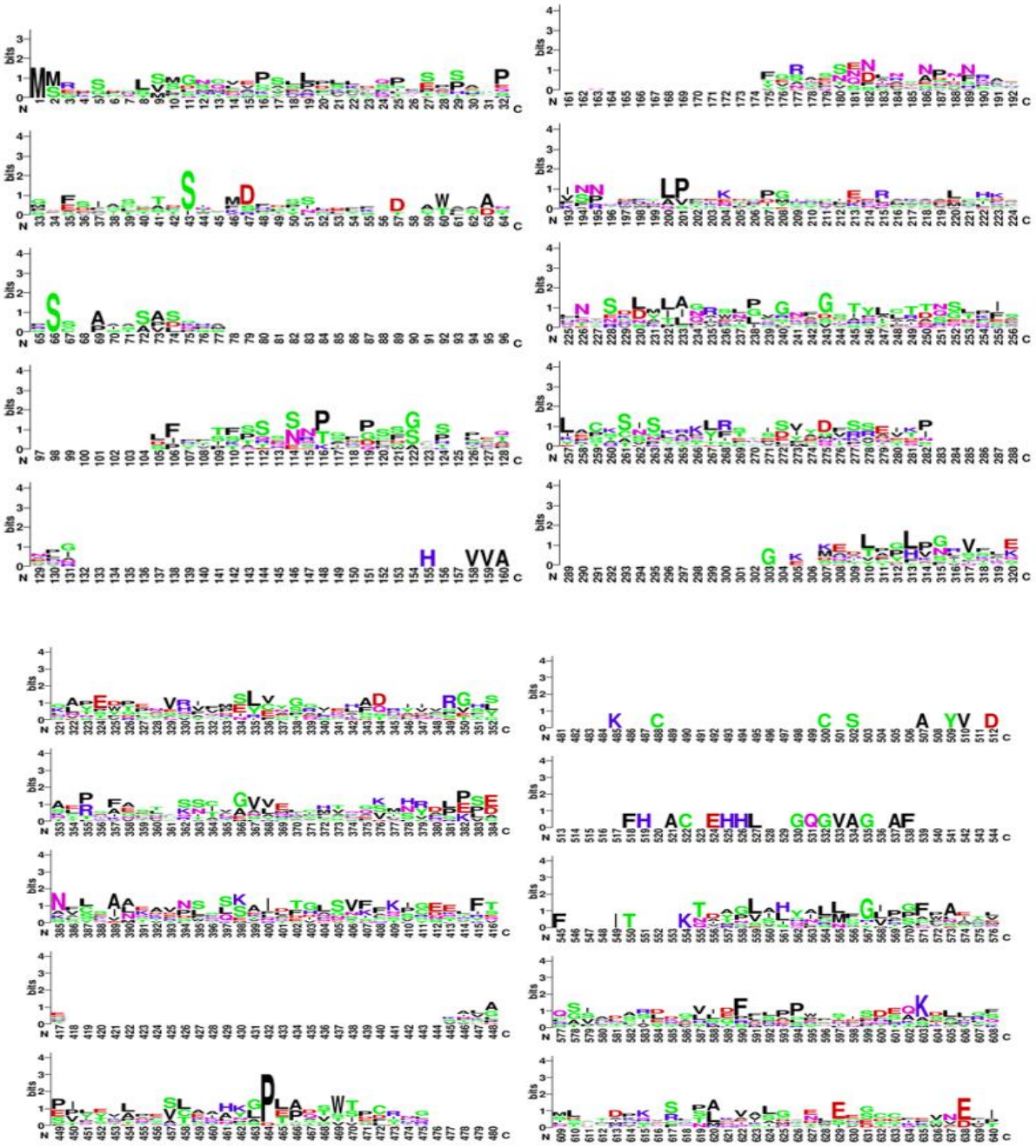


Fig: 8. Subcellular localization of all 15 proteins generated with Tbtool software.



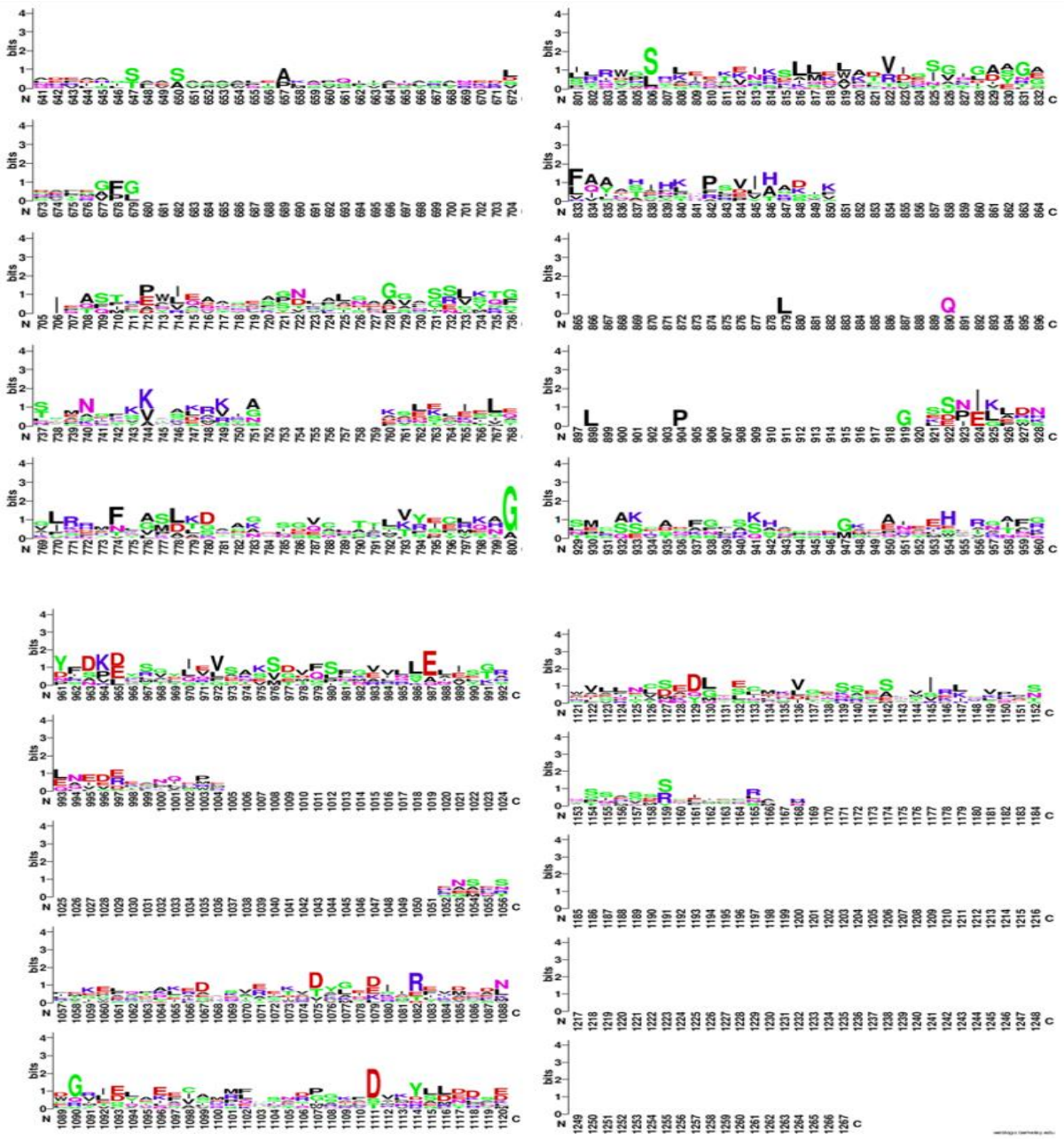
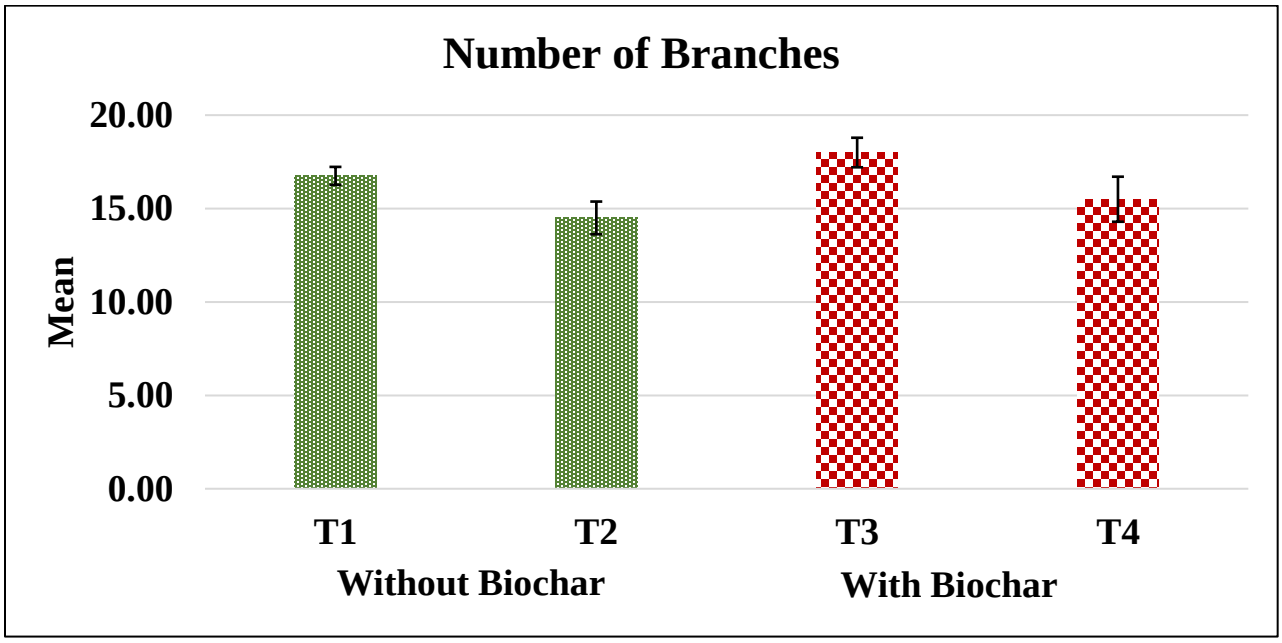


Fig: 9. Sequence logos analysis of amino acid sequences for all 15 genes. The N-terminal and C-terminal of NIG gene domain are indicated by using ‘N’ and ‘C’.

4.2. Wet lab Results

4.2.1 Number of Branches

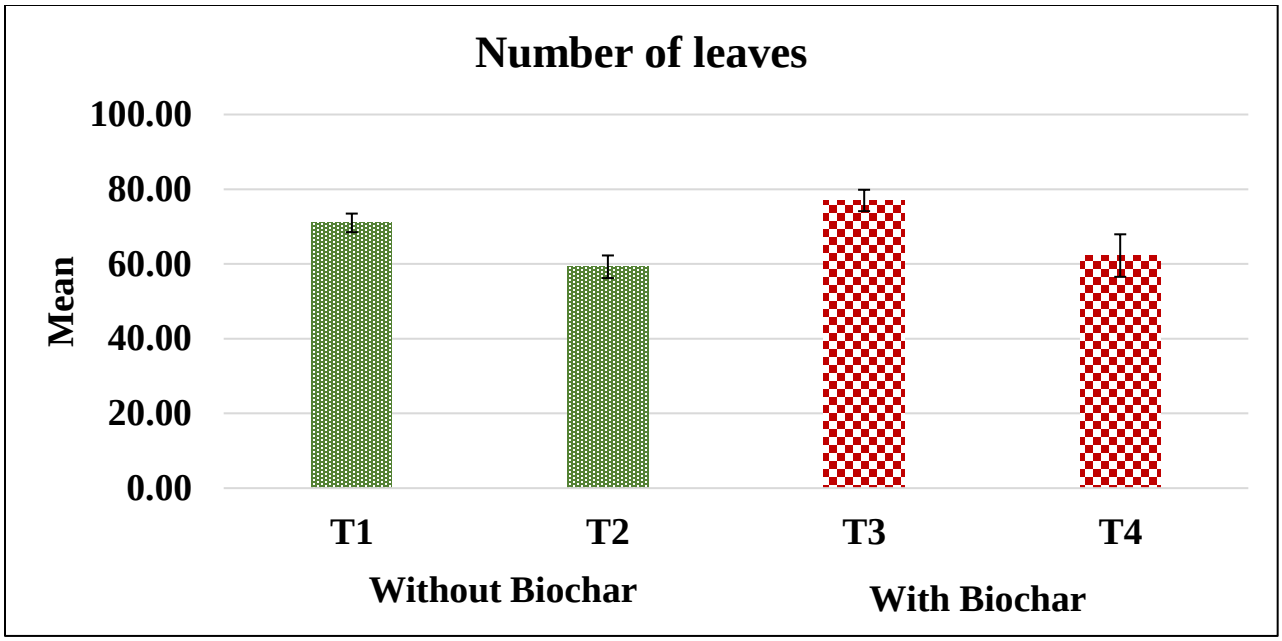
Number of branches were counted for each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea). Their mean valves, standard deviation, and percentage were calculated, along with *the p-value*. Results for the number of branches are shown here in the Bar graph: 1. Where on y-axis treatments are given and on X-axis mean value for the number of branches is given. Error bars are shown on top of each bar. The p-value for the number of branches is $p = 0.6497$ which indicates that results are not significant.



Graph: 1. Bar graph for number of branches is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.2 Number of Leaves

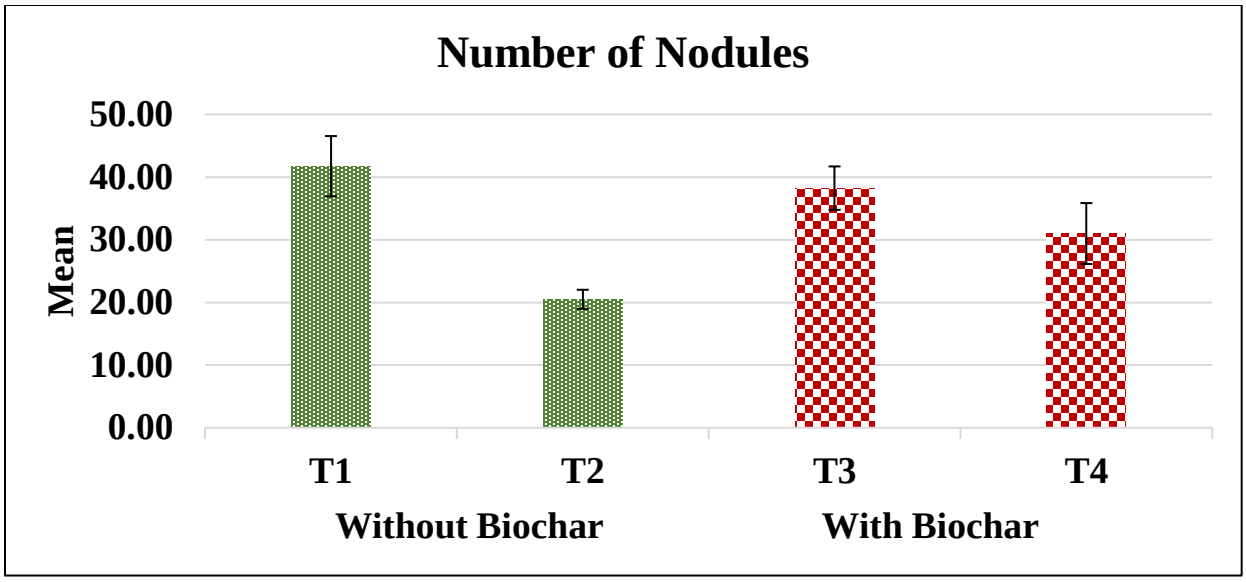
Number of leaves were counted for each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea). Their mean valves, standard deviation, and percentage were calculated, along with the p-value. The result for the number of leaves is shown here in the Bar graph: 2. Where on y-axis treatments are given and on X-axis mean value for the number of branches is given. Error bars are shown on top of each bar. The p-value for the number of leaves is $p = 0.4763$ which indicates that the results are not significant.



Graph: 2. Bar graph for number of branches is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.3 Number of Nodules

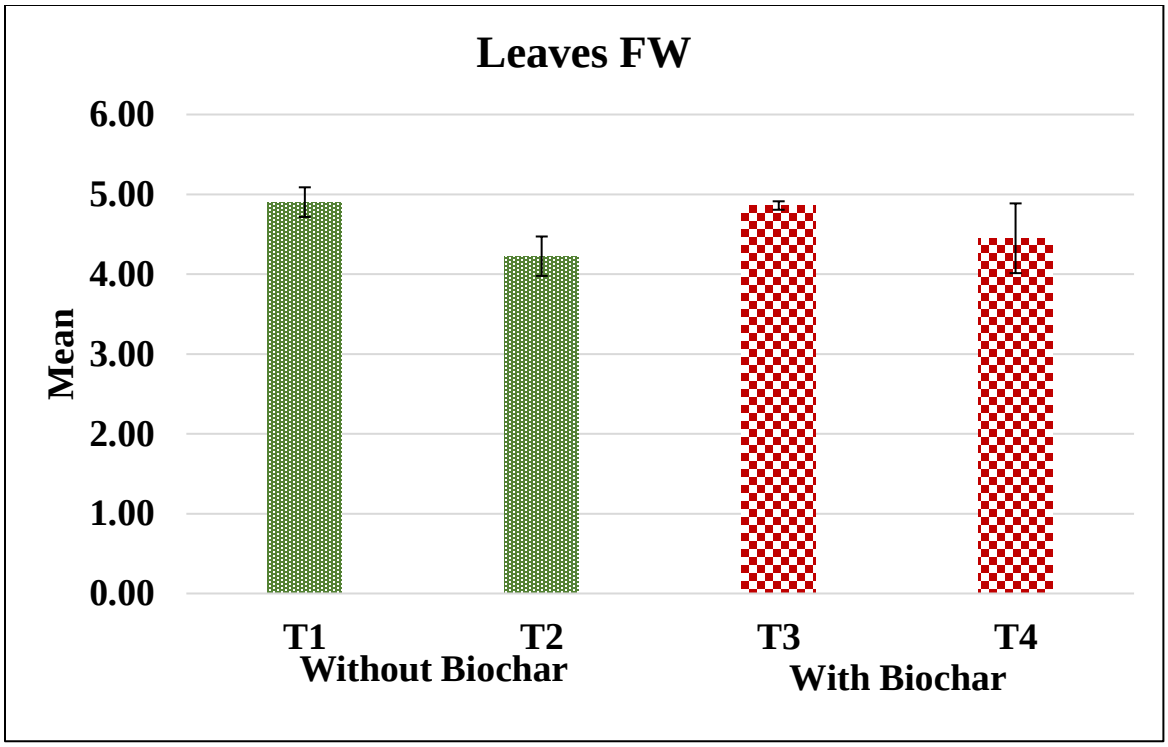
Number of nodules were counted for each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea). Their mean valves, standard deviation, and percentage were calculated, along with the p-value. The result for the number of nodules is shown here in the Bar graph: 3. Where on the y-axis treatments are given and on X-axis mean value for the number of nodules is given. Error bars are shown on top of each bar. The p-value for the number of nodules is $p = 0.3944$ which indicates that results are not significant.



Graph: 3. Bar graph for number of nodules is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.4 Leaves FW

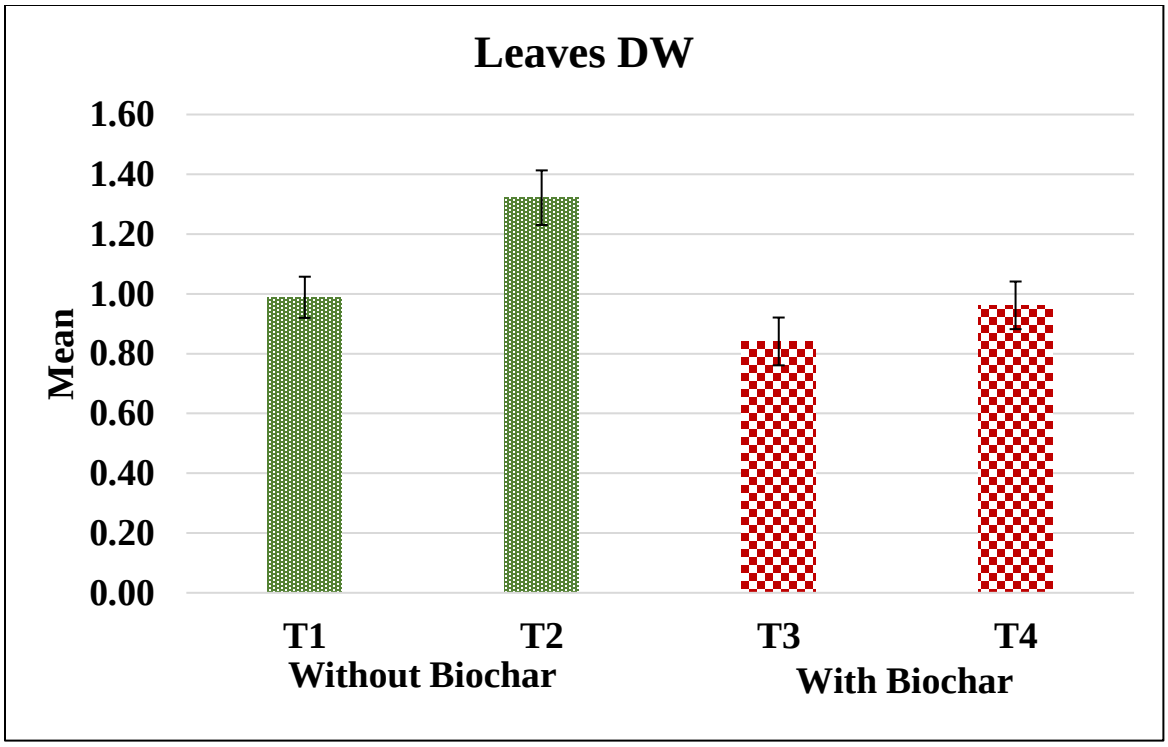
Leaves fresh weight was determined directly after harvesting each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea). Their mean valves, standard deviation, and percentage were calculated, along with the p-value. The result for the Leaves Fresh weight is shown here in the Bar graph: 4. Where on y-axis treatments are given and on X-axis mean value for Leaves FW is given. Error bars are shown on top of each bar. p-value for the number of nodules is p = 0.8399which indicates that results are not significant.



Graph: 4. Bar graph for leaves FW is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.5 Leaves Dry Weight

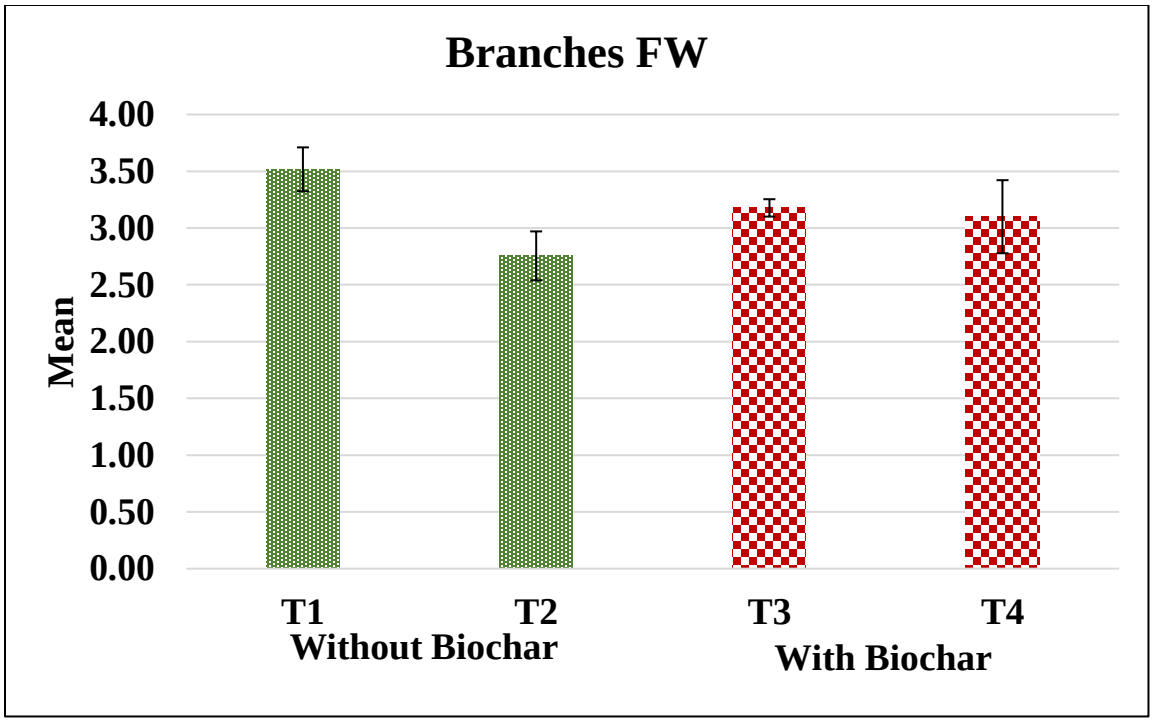
Leaves dry weight was determined after drying of leaves at 40C for each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea). Their mean valves, standard deviation, and percentage were calculated, along with the p-value. The result for the Leaves' dry weight is shown here in the Bar graph: 4. Where on y-axis treatments are given and on X-axis mean value for Leaves DW is given. Error bars are shown on top of each bar. the p-value for the number of nodules is p = 0.8399which indicates that results are not significant.



Graph: 5. Bar graph for leaves DW is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.6 Branches Fresh Weight

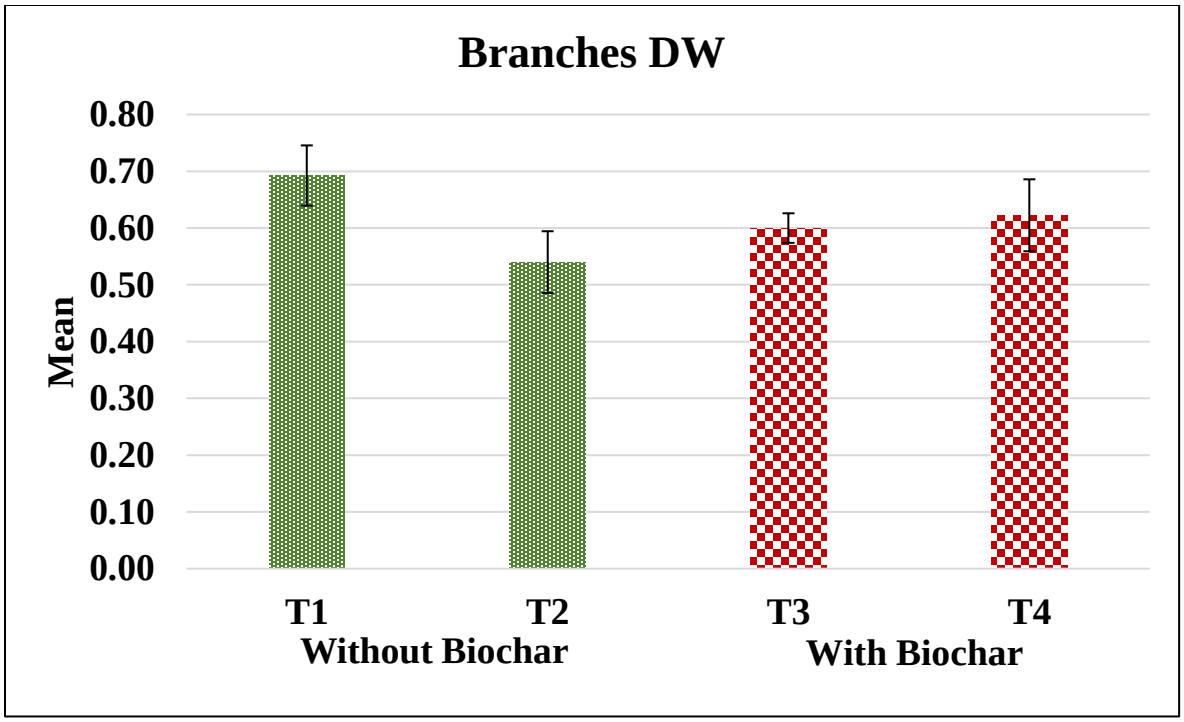
Branches Fresh weight was determined directly after harvesting each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea). Their mean valves, standard deviation, and percentage were calculated, along with the p-value. The result for the Branches' weight is shown here in the Bar graph: 6. Where on y-axis treatments are given and on X-axis mean value for Branches FW is given. Error bars are shown on top of each bar. The p-value for Branches FW is $p = 0.7691$ which indicates that results are not significant.



Graph: 6. Bar graph for Branches FW is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.7 Branches Dry Weight

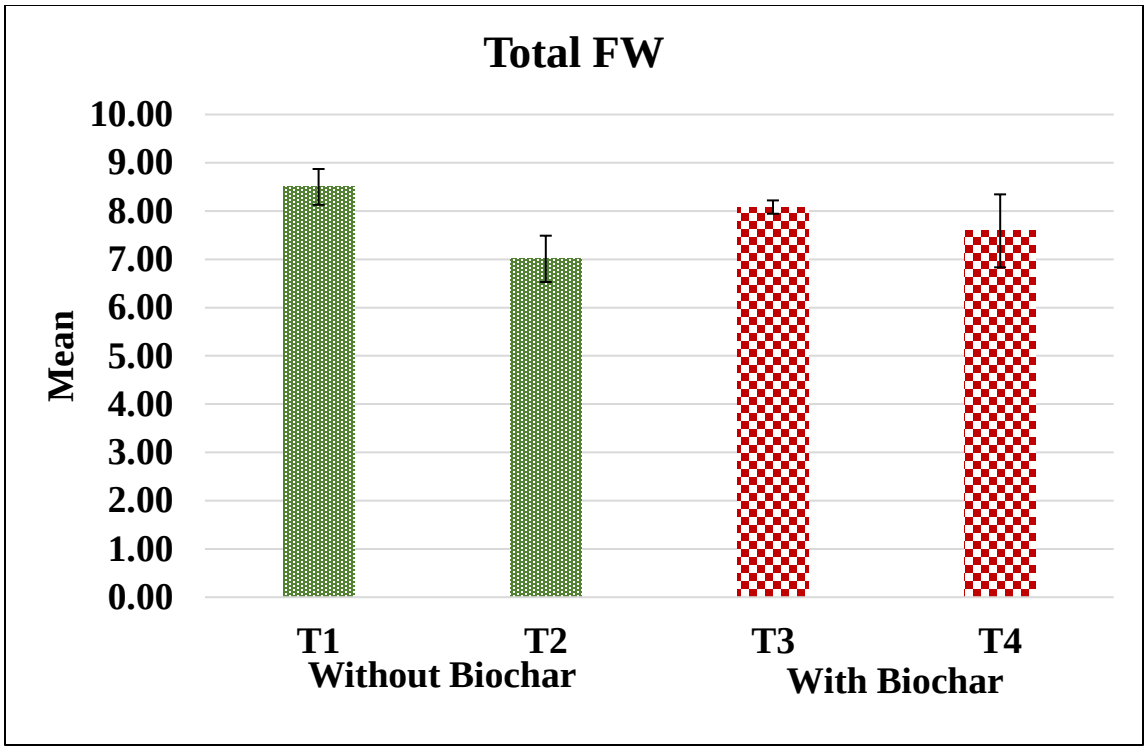
Branches Dry weight was determined directly after harvesting each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea). Their mean valves, standard deviation, and percentage were calculated, along with the p-value. The result for the Branches' dry weight is shown here in the Bar graph: 7. Where on y-axis treatments are given and on X-axis mean value for Branches FW is given. Error bars are shown on top of each bar. The p-value for Branches FW is $p = 0.8358$ which indicates that results are not significant.



Graph: 7. Bar graph for Branches DW is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.8 Total Fresh Weight

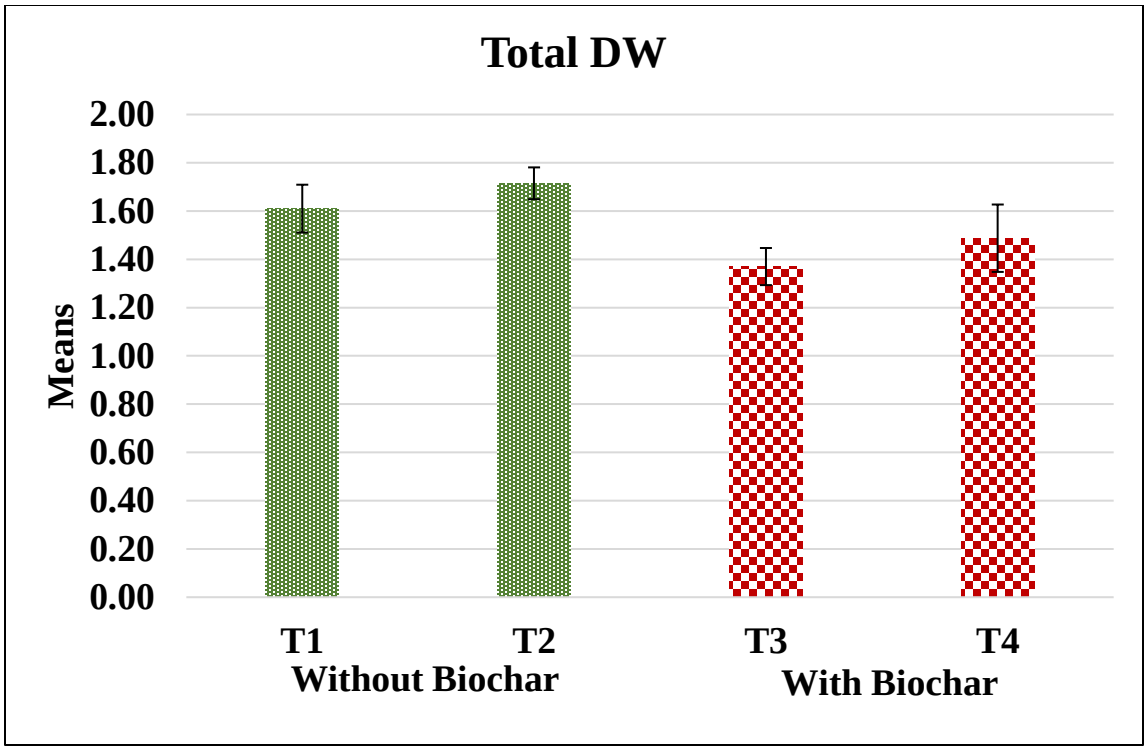
Total fresh weight was determined directly after harvesting each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea) via digital balance. Their mean valves, standard deviation, and percentage were calculated, along with the p-value. The result for the total fresh weight is shown here in the Bar graph: 8. Where on y-axis treatments are given and on X-axis mean value for total FW is given. Error bars are shown on top of each bar. The p-value for Branches FW is $p = 0.8089$ which indicates that results are not significant.



Graph: 8. Bar graph for Total FW is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.9 Total Dry Weight

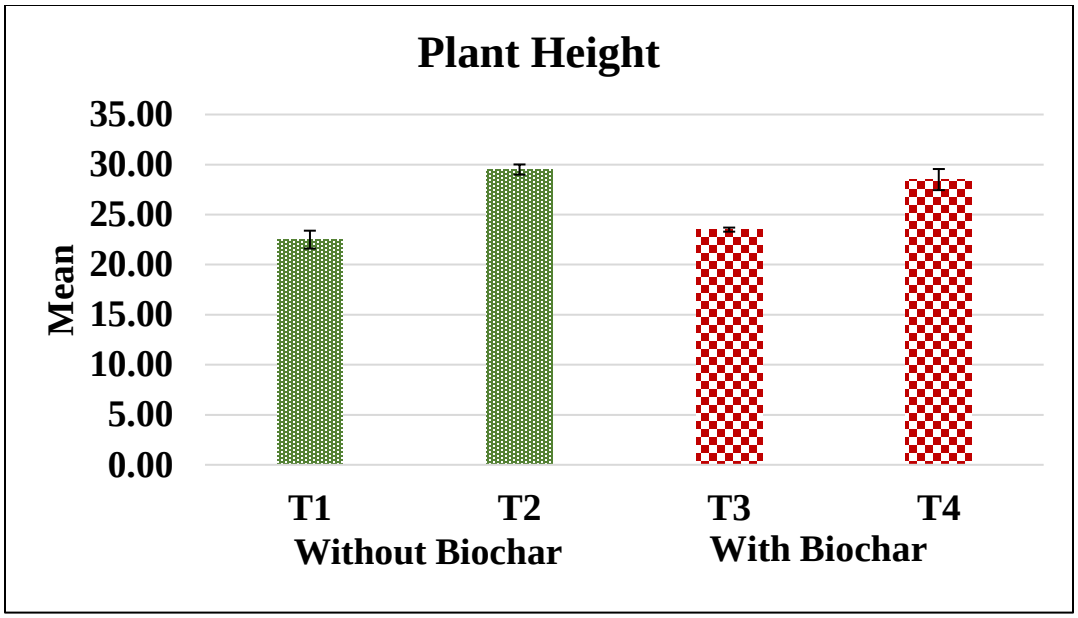
Total Dry weight was determined after dry plant sample for each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea) via digital balance. Their mean valves, standard deviation, and percentage were calculated, along with *P-value*. The result for the total fresh weight is shown here in the Bar graph: 9. Where on y-axis treatments are given and on X-axis mean value for total FW is given. Error bars are shown on top of each bar. The p-value for Branches FW is p = 0.3714 which indicates that results are not significant.



Graph: 9. Bar graph for Total DW is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.10 Plant Height

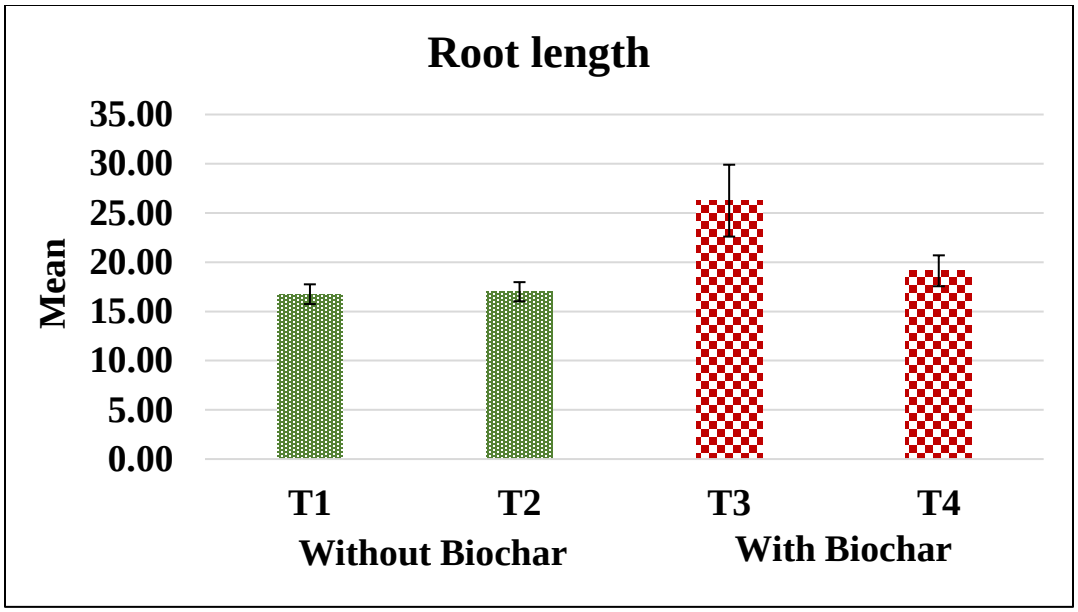
Plant height was determined for each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea) via digital balance. Their mean valves, standard deviation, and percentage were calculated, along with the p-value. The result for the Plant height is shown here in the Bar graph: 10. Where on y-axis treatments are given and on X-axis mean value for total FW is given. Error bars are shown on top of each bar. The p-value for Branches FW is $p = 0.8669$ which indicates that results are not significant.



Graph: 10. Bar graph for Plant height is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.11 Root Length

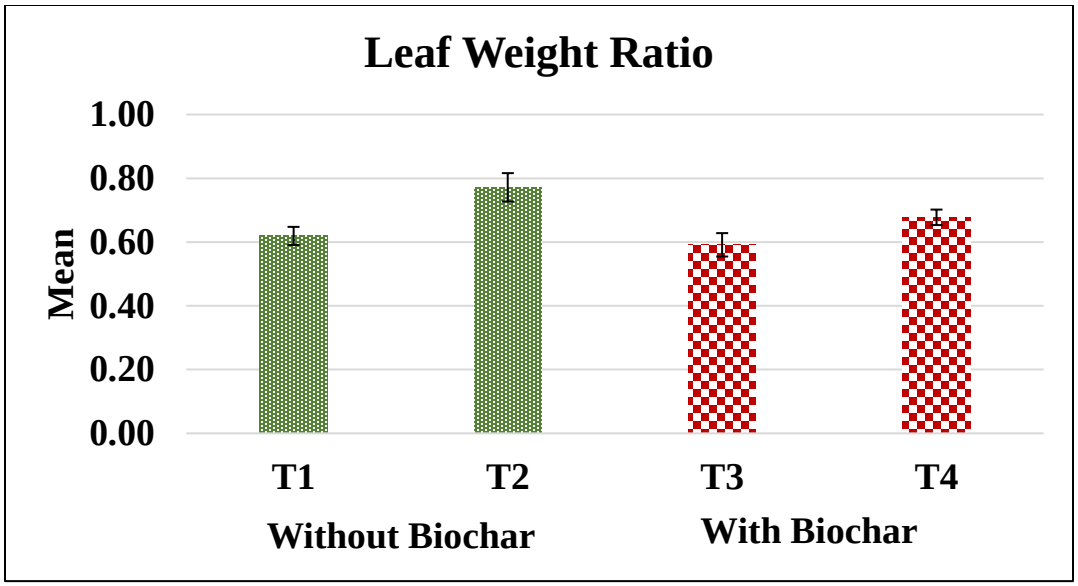
Root length was determined for each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea) via meter ruler. Their mean valves, standard deviation, and percentage were calculated, along with the p-value. The result for the root length is shown here in the Bar graph: 11. Where on y-axis treatments are given and on X-axis mean value for total FW is given. Error bars are shown on top of each bar. The p-value for Branches FW is $p = 0.5004$ which indicates that results are not significant.



Graph: 11. Bar graph for root length is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.12 Leaf Weight Ratio

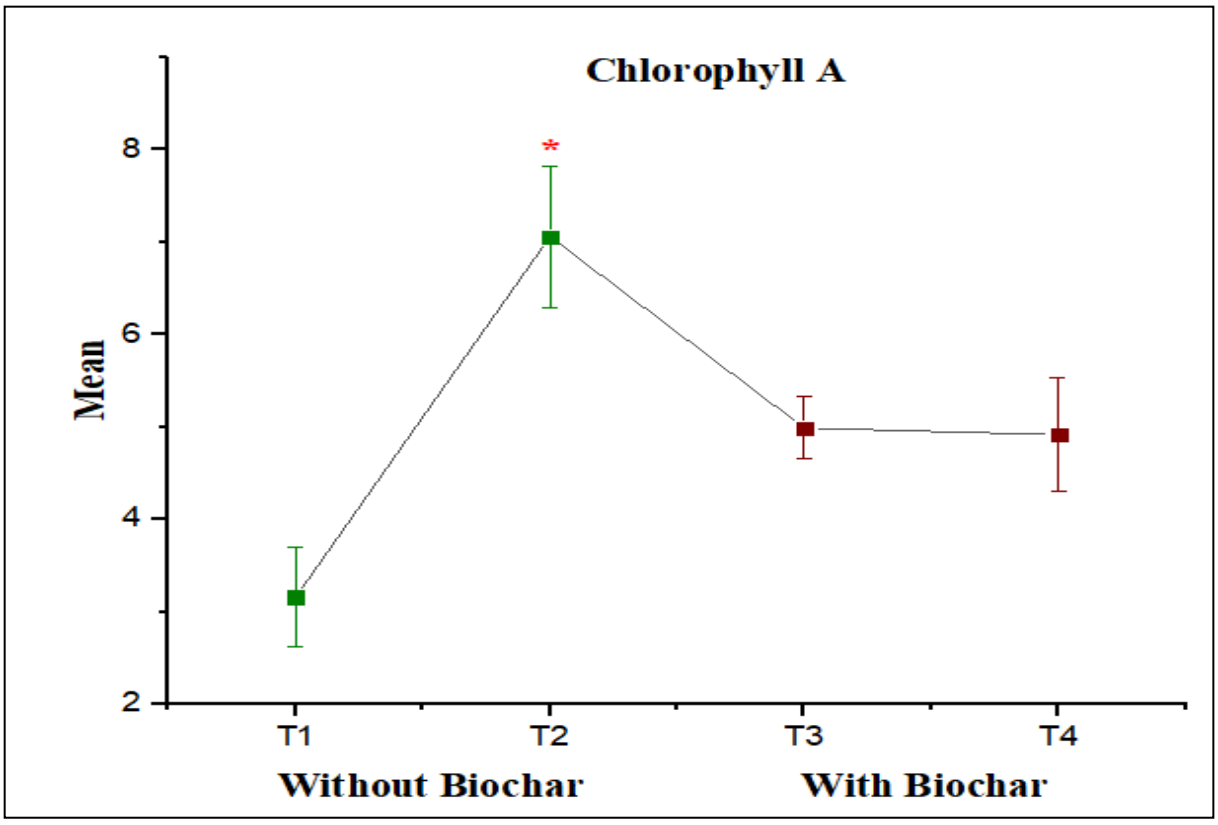
Leaf weight ratio was determined for each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea). Their mean valves, standard deviation, and percentage were calculated, along with *the p*-value. The result for the leaf weight ratio is shown here in the Bar graph: 12. Where on y-axis treatments are given and on X-axis mean value for leaf weight ratio is given. Error bars are shown on top of each bar. The p-value for Branches FW is $p = 0.4235$ which indicates that results are not significant.



Graph: 12. Bar graph for leaf weight ratio is given where green color bars represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted bars represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.13 Chlorophyll A Concentration

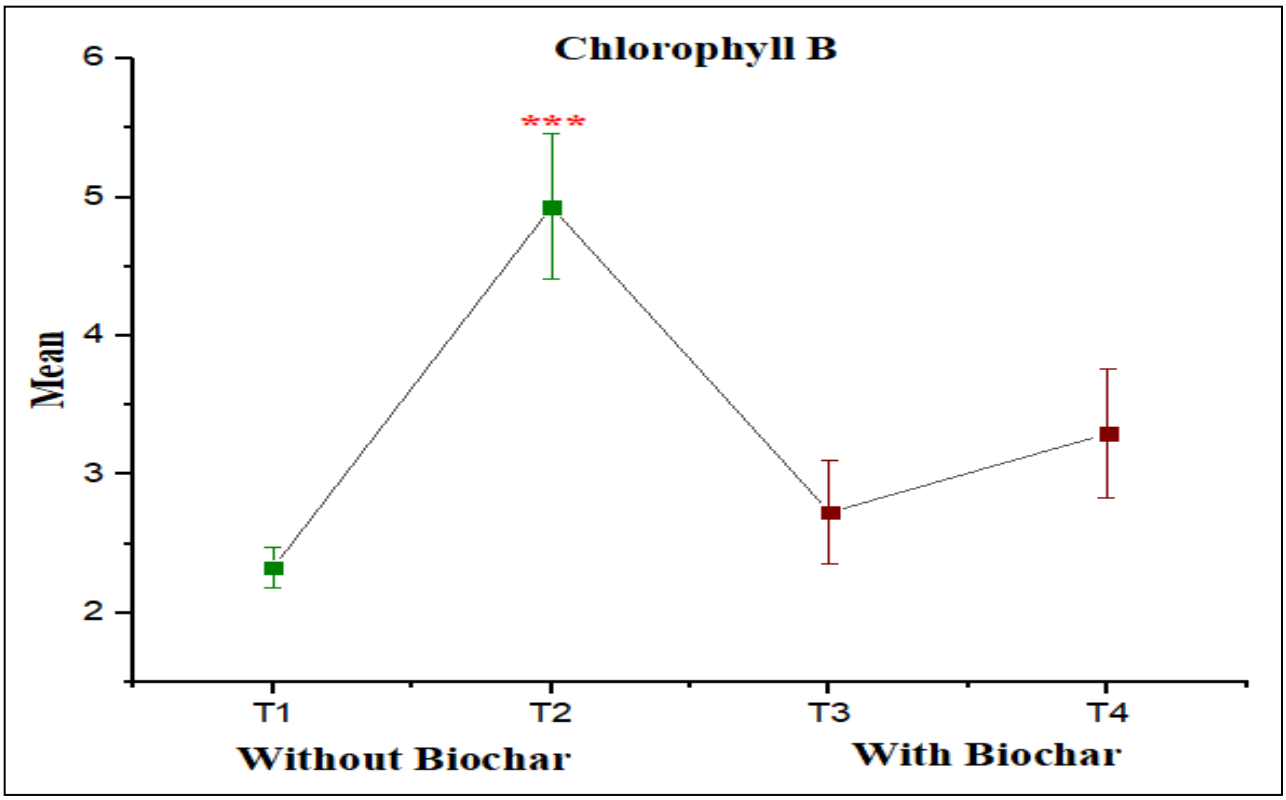
Chlorophyll A concentration was determined for each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea). Their mean valves, standard deviation, and percentage were calculated, along with *P-value*. Result for the chlorophyll A concentration is shown in graph: 13. Where on y-axis treatments are given and on X-axis mean value for chlorophyll A concentration is given. Error bars are shown on top of each bar. The p-value for Branches FW is $p = 0.00459$ which indicates that results are significant.



Graph: 13. Graph for chlorophyll A concentration is given where green color dots represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted dots represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

4.2.14 Chlorophyll B Concentration

Chlorophyll B concentration was determined for each treatment (T1 = control, T2 = Urea, T3 = Biochar, T4= Biochar + Urea). Their mean valves, standard deviation, and percentage were calculated, along with the p-value. The result for the chlorophyll B concentration is shown in graph: 14. Where on the y-axis treatments are given and on X-axis mean value for chlorophyll B concentration is given. Error bars are shown on top of each bar. The p-value for Branches FW is $p = 0.00334$ which indicates that results are significant.



Graph: 14. Graph for chlorophyll B concentration is given where green color dots represent without biochar treatments i.e., T1 for control and T2 for urea. Red color dotted dots represent treatments with biochar i.e., T3 for Biochar and T4 for Biochar + Urea.

Plant growth and development parameters showed non-significant results which indicate that urea with or without biochar is not affecting leguminous plants like *Arachis Hypogaea*. The nitrogen source provided in the experiment is not needed by the plant as biological nitrogen fixation is fulfilling the required nitrogen. Provided nitrogen quantity in the experiment is the recommended dose for the farmers (30 kg/hectare) that cost Rs 1700/-. Results suggested that the use of nitrogen fertilizer (Urea) in the peanut field is unnecessary and a waste of poor farmer money. Urea has harmful effects like soil and water pollution and causes global warming as it converts into NO3- and enters the atmosphere. However, the use of biochar might reduce the harmful effects of urea as biochar has the property of retention soil nutrients and water. Biochar is the permanent source of carbon in the soil compared to urea which leach out from the soil. Chlorophyll content on the other hand is significantly affected by urea which reveals the direct relationship of nitrogen fertilizer with chlorophyll content. Chlorophyll has nitrogen that might be the reason for the maximum concentration of chlorophyll in the presence of urea fertilizer.

Chapter 5: Discussion and Conclusion

Arachis hypogaea gained huge botanical attention in recent years than other plants. It is an important legume crop having 48-50% oil content and 25-28% protein. Active flavonoids are also found in abundant amounts. Peanut kernels produces 5667 kcal energy per 100g (Jambunathan 1991). Furthermore, for boosting immunity and prevention of lymphatic disorder peanut could be used. Oligomeric procyanidins and resavtrol presence in peanuts make it useful in the prevention of cancer cells formation. Due to the high smoking point, peanut oil is a good cooking medium. Shells of the peanut seeds are used as organic fertilizers, making boards and in the feed industry (Arya et al. 2016). The symbiotic relation of Arachis hypogaea and plant growth-promoting rhizobium is the reason for biological nitrogen fixation. The plant provides shelter and food to rhizobia and in return rhizobia fix atmospheric nitrogen for plants.

Bioinformatic study on all nodulation genes is not conducted in Arachis hypogaea in past. Therefore, 15 genes functioning in the nodulation process were identified by using query sequences from other leguminous plants. These genes were named in NIG family and genome-wide analysis distributed these genes in three sub-families NIG-a, NIG-b, and NIG-c having 6, 7, and 2 genes, respectively. Their role in nodulation was confirmed by the identification of domains and their function. Furthermore, biophysical properties showed their different roles in the symbiotic pathways. 543 bp- 2958 bp is the range of the CDS region and amino acid residues range from 559-986. The range of isoelectric point and molecular weights are 4.82-6.7 and 62975.18-109443.61 respectively. Structural analysis reveals that all genes are full-length genes with upstream intronic regions. Motif analysis confirmed ten conserved motifs in all genes with a signature motif 8 having “EKLTTRPEVEDVRRELEAMNL” region which indicates similarity among the structure of genes. Motif identification and gene structure validated the tree classification. Chromosomal distribution of the genes was uneven with two homologous gene pairs generated by segmental duplication. Segmental duplication occurs between 0.28674973 and 0.46897178 MYA. Moreover, subcellular localization results located three sites for protein i.e., cell membrane, nucleus, and chloroplast.

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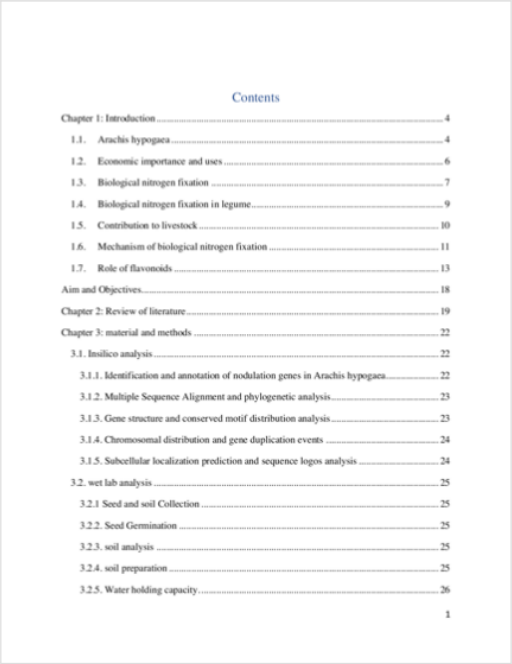

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1

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