Organic amendments and ammonium sulphate impact on nitrogen fixation and flavonoids in *Arachis hypogaea*

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DEDICATED TO MY LOVING PARENTS AND INCREDIBLE HUSBAND WHO SUPPORTED ME AND BELIEVED IN ME.

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Anum Akram

Abbreviations

AI	Aliphatic index
BARI	Barrani agriculture research institute
cDNA	copy of DNA
ESTs	Expressed sequence tags
FAO	Food and Agriculture Organization
NCBI	National Centre for Biotechnology Information
MEGA	Molecular Evolutionary Genetic Analysis
ORF	Open Reading Frame
NIG	*Nod* Inducing Gene family
GRAVY	Grand average of hydropathicity
(NH4)2SO4	Ammonium Sulphate
SOC	Soil organic carbon
NJ	Neighbor Joining
Ks	Synonymous
Ka	Non-synonymous

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Abstract

Legume plants have an additional ability to form associations ranging from largely nonspecific to very specific interactions. Within the nodules i.e. the new organs developed in host plants, selective rhizobial colonization is lead by a complex signal exchange. However, these complex signals and nodulation mechanism is highly specific as it involves several *Nod* inducing gene families. These genes are involved in several stages of biological nitrogen fixation which includes flavonoids exudation from roots and perception on rhizobacteria, phosphorylation cascade of protein kinases, node genes expression in rhizobacteria, node proteins causing root hair curling and nodule formation. Here computational and bioinformatics analysis of *Nod* inducing genes performed so that to explore their potential functions in the *Arachis hypogaea* for the first time. In this study, a genome-wide analysis of 12 *Nod* inducing genes in *Arachis hypogaea* done followed by phylogenetic clustering analysis, gene structure determination, detection of conserved motifs, subcellular localization and conserved motifs based on a homology study of genes of some other legume plant. And finally, unraveled the potential involvement of these genes in nodulation and biological nitrogen fixation.. Results showed that the motifs are highly conserved and all the genes are full-length genes with upstream intronic regions. Thus study not only provides identification and characterization of genes underlying developmental and functional stages of nodulation and biological nitrogen fixation but also find application of ammonium sulphate in combination with biochar may prolong nitrogen availability as per plant demand. In the present study, application of ammonium sulphate with and without biochar found no significance results like in biomass production which suggests ammonium sulphate applied at 30kg per hectare is not environment friendly approach. Besides, identification and structural analysis of these genes in Arachis hypogaea may provide a theoretical basis for the study of evolutionary relationships in future analysis.

Keywords: *Arachis hypogaea*, Bioinformatics, Phylogeny, Gene Duplication, Cis-elements, Sequence logos, Phylogenetic analysis, Chlorophyll analysis

Chapter 1

Introduction

1.1. Arachis hypogaea

Groundnut (*Arachis hypogaea*) is an important legume, oilseed self-pollinated crop belonging to the Fabaceae family also known as Leguminosae. *Arachis hypogaea* is a typical variety of genus *Arachis* with a long cycle, absence of flowers on the central stem, and alternating vegetative and reproductive side stems. The plant measure about 30-50 cm in length. Leaves of Peanut plants are alternate and pinnate having four leaflets with no leaflet at the terminal. The leaflet has length and width 1-7 cm and 1-3 cm respectively. The flower of Peanut is bright yellow in color and borne in axillary clusters above ground. The flower lasts for one day only. The ovary of the plant is at the base which seems to be a flower stem but is an elongated floral cup. After pollination of flowers, a short thick stem at the base of flower termed, gynophore, grows in the downward direction and penetrates in the soil. A thin brown colored paper-like seed coat covers the seed. The root in the peanut plant is a well-developed taproot with several lateral roots extending several inches into the ground. Most of the roots have nodules (Suchoszek-Łukaniuk et al. 2011).

Peanuts are cultivated from 40°S to 40°N latitude and the appropriate regions for its growth and cultivation include tropical, sub-tropical, and warm temperate zones. Peanut is sensitive to frost and the period required for its growth is about 4–5 months. The type of land required for peanut growth is heavy soils but can also be grown in light, dusty, and having less water sandy loams. The annual mean temperature, rainfall, and pH which can be tolerated by peanut is 10.5°C to 28.5°C, 3.1 to 41.0 dm, and pH of 4.3 to 8.7, respectively. These annuals were determined based on their growing zones which range from tropical thorn (cool temperate moist) to regions of wet forest. The plant grows further and produces around 20-40 pods at maturity (Duke 2012).

Fig:1.1. 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.Taxonomy

Groundnut (Arachis hypogaea L.) is one of the most important oilseed self-pollinated crops. It belongs to the family Leguminosae (ancient name) Fabaceae (current name) and subfamily Papilionaceae. *Arachis hypogaea* Linn is derived from two Greek words, *Arachis* meaning a legume and *hypogaea* meaning below ground, which refers to the formation of fruits (pods) in the soil. *Arachis hypogaea* is allotetraploid (AABB 2n = 4x = 40chromosoms) with two main classes; *fastigiata* and *hypogea* (Leal-Bertioli et al. 2012).

The classification of peanut is given below:

Kingdom	Plantae
Subkingdom	Viridiplantae
Infra kingdom	Streptophyta (Land plants)
Super division	Embryophyta
Division	Tracheophyta (Tracheophytes, vascular plants)
Subdivision	Spermatophytina (Seeds plants, sermatophytes)
Class	Magnoliopsida
Superorder	Rosanae
Order	Fabales
Family	Fabaceae (legumes and peas)
Genus	Arachis L.
Species	hypogaea
Peanut Plant	Arachis hypogaea L.

(Lokesh et al. 2019)

1.3. Arachis hypogaea economic and agricultural importance

Peanut has very high commercial, nutritional and agricultural value. Peanut is cultivated in around 100 countries of the world as a legume crop and a source of oil, food, and cattle feed. It was reported that from worldwide groundnut production, about 60% is used for industrial uses and edible oil extraction while 40% is used for the food and seed reservoir for next season sowing of the crop (Sardar et al. 2017). In Asia, 35% of the peanut production is consumed as food while in America 75% is used for the same purpose. Peanuts can help to enrich the soil. Peanuts are legumes and are able to fix nitrogen in their roots and their oil used as a liniments, plasters, soap & lubricant (Karra

et al. 2013). For livestock, peanut haulms constitute nutritious fodder. They contain carbohydrates (38-45%), minerals (9-17%), protein (8-15%), and lipids (1-3%) at levels higher than cereal fodder.

One of the inexpensive and most widely used vegetable oil is peanut oil, which is not only used for nutritional purposes but also for fuel and radiance. The calories present in peanut oil are 549 cals/100 g, according to of WOI and which is five times more than that of beef. Due to Peanut oil's high-level smoking point, it is an outstanding medium for cooking (Yusuf et al. 2017). Peanut seed flour is used to make baked products and confections as well as seeds are consumed as roasted, raw, or boiled. The stalk of peanut is used as fodder for animals, as silage or green fertilizer. The shells of the peanut are used as filler in fertilizers, as fuel, for making particleboard, and in the feed industry. The energy production by peanut kernels is 564 kcal per 100 g (Pasupuleti et al. 2013). After extraction of oil, the cake is obtained which is used as feed in the cattle industry, as a soil amendment, and used to make digestible food for aged persons and children.

The coverage area for peanut in the world is 24 million hectares with 38 million tons' production. In the last few years, groundnut production has shown a significant increase. The increase in production of peanut was 0.1% while the increase in peanut cultivation area was 0.3% (Pasupuleti and Nigam 2013).A thin brown colored paper-like seed coat covers the seed. Being a legume crop, through biological nitrogen fixation, peanut helps to improve soil fertility. The root in the peanut plant is a well-developed taproot with several lateral roots extending several inches into the ground. Most of the roots have nodules. The plant has the ability to fix atmospheric nitrogen, due to nodulation they can fix maximum nitrogen from the soil and do not require fertilizers containing nitrogen. Due to the ability of nitrogen fixation, they effectively increase the fertility of the soil. This feature makes peanut an important crop in crop rotations. The crop rotation also increases the yield of peanut as the risk of pest attack and disease is reduced. Apart from that, the soil must contain sufficient levels of phosphorus, potassium, calcium, magnesium, and micronutrients.

1.4. Nutritional value of Arachis hypogea

Like all legumes, groundnuts are low in sodium and are good sources of magnesium, calcium and potassium, a group that is associated with reduced risk of cardiovascular

diseases (Arya et al. 2016). Peanut has a high nutritional value as it is an important source of numerous secondary metabolites like flavonoids, resveratrol and pterostilbene which play an important role in plant defence mechanisms (Goyal et al. 2012). Moreover, it is an important source of vegetable oil (50%), protein (25%) and plant secondary metabolites like flavonoids, isoflavones, polyphenols, tocopherols, folic acid, and resveratrol (Wang et al. 2013). It serves as a dietary source of vitamin E and phytosterols. Many health-improving nutrients for example, minerals, antioxidants, vitamins, and fatty acids (monosaturated) are constituents of groundnut kernels. It has varies pharmacological activities like antimicrobial, antifungal, antiviral, antioxidant, anticancer, antihypertensive, neuroprotective, antimutagenic, antiproliferative, anti-inflammatory. Though, extensive knowledge about flavonoids biosynthesis pathway genes has been studied in Grape, Arabidopsis, and Petunia (Falcone Ferreyra et al. 2012). Nuts are beneficial due to the presence of desirable lipid profile, although peanut is a legume but contain high amount of unsaturated fatty acids (Falcone Ferreyra et al. 2012). Peanut, in addition to beneficial nutrients, contains various bioactive compounds which provide health benefits not only to plants but to human health upon their consumptions (Lokesh et al. 2019).. These compounds have disease preventive characteristics and promote the health of their consumers (Karra et al. 2013). Total composition of peanut is given in table 1.1.

Table:1.1. Composition of Groundnut.

Nutrient	*Unit*	*Per 100 gram*
Water	g	6.5
Energy	Kcal	567
Fats	g	49.24
Saturated	g	6.279
Monosaturated	g	24.426
Polysaturated	g	15.558
Cholesterol	g	0
Carbohydrate	g	16.13
Fiber	g	8.5
Sugar	g	4.72
Protein	g	25.80
Tryptophan	g	0.25
Threonine	g	0.883
Isoleucine	g	0.907
Leucine	g	1.672
Lysine	g	0.926
Methionine	g	0.317
Cystine	g	0.331
Phenylalanine	g	1.377
Tyrosine	g	1.049
Valine	g	1.082
Magnesium	mg	168
Iron	mg	4.58
Calcium	mg	92
Zinc	mg	3.27
Potassium	mg	705
Phosphorus	mg	376
Manganese	mg	1.934
Copper	mg	1.144
Proanthocyanidin dimers	mg	33.2
Proanthocyanidin trimers	mg	48.8
Proanthocyanidin 4-6mers	mg	48.1
Thiamine (B1)	mg	0.64
Riboflavin (B2)	mg	0.135

1.5. Nitrogen fixation and Flavonoids in *Arachis hypogaea*

Nodulation and biological nitrogen fixation could be modified by co-inoculation of plant growth promoting bacteria with rhizobacteria. Plants form a new organ called nodule in the roots by a mutually beneficial process known as root nodule symbiosis. A nodule in roots is a structure where rhizobacteria hosted and convert atmospheric nitrogen into ammonium and deliver it to the plant. This nodulation depends on expression patterns of nodulation related genes and the expression of these genes greatly varies with different soil conditions (Lagunas et al. 2019). Plant-microbial symbiosis is dependent on underground communications, however, essential nutrients like nitrogen and soil organic matter may influence this relationship.

Flavonoid, a key signal in plant-microbe symbiosis, released from roots (as root-exudates) is recognized by rhizobia in the soil. The perception of flavonoid leads to the expression of nod genes for node factors (lipo-chitooligosaccharides) synthesis (Falcone Ferreyra et al. 2012). Flavonoids solubilize phosphorous in the soil and reduce the need for phosphorous up to 50%. It dissolves phosphatase into phosphorous and iron. Flavonoids provoke transcription factors of the genes in the rhizobacteria to trigger the synthesis of Nod factors, these Nod factors are then perceived by plants specific receptors to allow symbiotic infection of roots, leading to nodule formation (Skorupska et al. 2017). Nodulation related genes respond to certain environmental conditions which include different soil conditions. Therefore, understanding differential expression patterns is of crucial importance for high nitrogen fixation potential. Because Biological nitrogen fixation is environmentally friendly and could enhance sustainable agriculture.

The flavonoid signaling in rhizosphere is hindered by different soil constituents such as nitrogen and organic matter, leading to decreased nodulation and biological nitrogen fixation (Valle et al. 2020, Liu et al. 2020). With the increase of N supply in soil, exudation of flavonoids (Naringenin, hesperetin, and genistein) decreases from the roots (Liu et al. 2020). Nitrogen accumulation in different plant parts is inversely related with C/N ratio which influence flavonoids biosynthetic pathways and reduce flavonoids content in roots (Deng et al. 2019). Nitrates destroy indole acetic acid, decreasing lectin binding site by host plant that leads to the hinderance in the formation of root hair curling (infectious thread). Without infections thread formation in roots, attachment of

rhaizobia on roots is constrained. Even if nodules formation occurs, nitrate conversion to nitrite damage nodule and cause nodule senescence. Complex formation of leghemoglobin and nitrous oxide in root nodule results in the loss of leghemoglobin oxygen carrying ability and thereby BNF process is inhibited (Dogra and Dudeja 1993). Soil organic carbon (SOC) content of 2.03% to 3.80% is required for utmost inoculated Rhizobium growth in soil (Swanepoel et al. 2011). The high amount of organic carbon in the soil decreases flavonoid signaling up to 70% which leads to 75% reduction in root nodulation.

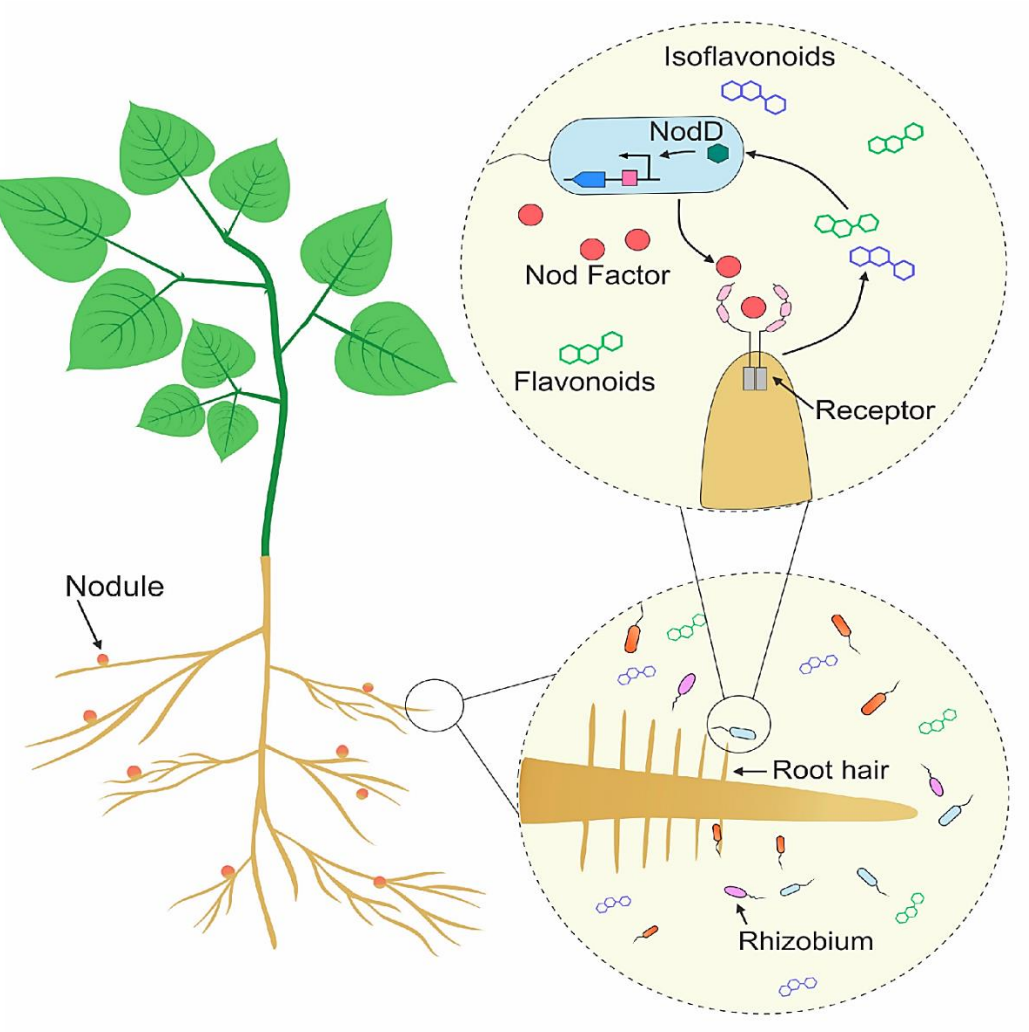

Fig:1.2. Nod factors are perceived by receptors present in the plasma membrane of root cells, triggering the signaling pathway required for the development and infection of the nodule, where bacteria are allocated, and nitrogen fixation occurs (Clúa et al. 2018).

1.6. Nitrogen-Based Chemicals and Organic Carbon

The selection and preparation of the suitable amount of nitrogen sources and organic matter that can aid in reasonable amount of flavonoid exudation and BNF. Shortly, will reveal a combination of nitrogen-based chemicals and organic carbon best for biological nitrogen fixation, plant growth, and development and might add value to the ecosystem (Koskey et al. 2017). As this product might have a low effect on biological nitrogen fixation, the plant will utilize symbioses for nitrogen uptake. Consequently, its usage will be low and promote cost-effectiveness in the agriculture industry. Fertilizers supply nutrient to plants and improve crop productivity and yield. They may be natural or industrially produced. Urea, Ammonium Sulphate, Calcium Ammonium Nitrate and Calcium nitrate are some of the nitrogen-based fertilizers. Urea production in Pakistan was 5.65 million tonnes in 2017. Ammonium sulphate import increased to 39,028 tonnes in 2018. Fertilizer treatment in Pakistan enhanced to 14-fold and nitrogen supply increased 20 kg to 133kg from 1970 to 2014. Furthermore, nitrogen fertilizer prices jumped 336% from 2001 to 2019. 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.

As expected, the nutritional state of the soil has a huge influence on the symbiosis, as well as the independent growth and survival of both plant and rhizobacteria. Changes in the chemical and physical properties of the soil subsequently influence its biological functioning and nitrifying communities.

1.6.1 Use of Biochar and Ammonium Sulphate:

The use of different types of nutrients is gaining popularity, especially in agriculture management. Such as the use of biochar in geographic areas where the addition of biochar to degraded soils can improve soil characteristics and therefore lead to higher crop yields. Biochar can absorb nitrogen, phosphorous, other nutrients, and N

fertilizers and release them through the cation exchange process (Horel et al. 2019). While contributing essential nitrogen for plant growth In the soil, the ammonium ion is released and forms a small amount of acid, lowering the pH balance of the soil. It contains 21% nitrogen and 24% sulfur (Sugimoto et al. 2021).

Peanut is an important legume crop of our country providing income to many people. The current research will help improve the nutritional value of crops and increase income for the country. The application of reasonable sources of ammonium sulphate and biochar to manipulate the root-rhizosphere interaction in the soil provides economical and impactful outcomes in agriculture sector as compared to currently available fertilizers. Local farmers and fertilizer industries will gain profitable benefits from the product and might influence the foreign exchange earnings when exported.

Aims and Objectives

The aim of the study is to analyze organic amendments and ammonium sulphate impact on nitrogen fixation and flavonoids in *Arachis hypogaea* with the given objectives.

1. Insilico identification and analysis of nodulation genes in peanut.
2. To identify the effects of ammonium sulphate fertilizer with or without application of biochar on growth and physiology of peanut

Chapter 2

Review of Literature

Peanut spread from USA to Europe and from there it reached Asia and Africa by the traders (Wright et al. 1994). By now, peanut has gained importance for poor-income farmers in Africa and Asia being as both cash and food crop. In 1940 to 1950, peanut was introduced and planted in Rawalpindi Division on 400 Ha. It is mainly grown in rain-fed areas known as barani areas. According to PARC, Punjab is the major contributor to peanut production while Sandhar, Sukkhar, Peshawar, Mardan, Kurram agency and Kohat are some of the major areas of its cultivation. Among legumes, peanuts are a good source of unsaturated fatty acids and contain various phytochemicals that positively affect human health (Bankole et al. 2005). According to an epidemiological survey, peanuts promote human health by lowering cholesterol and low-density lipoproteins due to the presence of beneficial fatty acids and phytochemicals (Alper and Mattes 2003).

Groundnuts are an outstanding also an inexpensive source of nutrition, providing essential nutrients to the human body such as carbohydrates, proteins, fibre, vitamins, lipids and minerals. Groundnuts, when taken in an acceptable amount, supplements human body with essential nutrients that can deliver energy, facilitates growth, and plays a significant role in the prevention of diseases (Settaluri et al. 2012). It is considered as the 3rd most important vegetable protein source and contains 50% edible oil, 20% carbohydrates and 28% digestible protein (Bhatti and Soomro 1994). Groundnuts are the richest nut sources of folate. Groundnuts, like other legumes, are low in sodium and good sources of calcium, magnesium and potassium, a group that is associated with reduced risk of CVD (cardiovascular diseases) (Higgs 2003). Groundnuts contain important amino acids that are required for proteins synthesis. Carbohydrates are present in adequate amount in groundnuts as they have a fundamental role in providing valuable nutrition to human body. Hence groundnuts along with groundnut oil can serve as a healthy form of nutrition. Groundnuts are a vital source of water-soluble vitamins for the human body along with vitamin E which is fat-

soluble. Another important vitamin that is supplemented by the intake of groundnuts is vitamin B3 (known as Niacin), to an extent of 13.525 mg (Settaluri et al. 2012). Commercially groundnuts are used mainly for oil production as they contain high oil content and is listed under important oil seed crops. In recent studies, it is revealed that groundnuts are also a good source of many bioactive components including phenolic acids, phytosterols, resveratrol and flavonoids. In plant and food products, bioactive compounds are present in small quantities as an extra nutritional constituents that occur naturally (Kris-Etherton et al. 2002). Groundnuts are a rich source of flavonoids and their dietary intake is recommended to combat different diseases. These bioactive components are known for their antioxidant properties, disease preventive properties and are thought to promote long life.

Secondary metabolites are considered to have no fundamental role in basic plant life processes. However, the idea behind the functioning of their role in stress response is largely supported by scientists (Agati and Tattini 2010; D'Auria and Gershenzon 2005; Winkel-Shirley 2002). Plant-microbial symbiosis is dependent on under-ground communications, however, essential nutrients like nitrogen and soil organic matter may influence this relationship (Shah and Smith 2020; Valle et al. 2020). Flavonoid, a key signal in plant-microbe symbiosis, released from roots (as root-exudates) is recognized by rhizobia in the soil. The perception of flavonoid leads to the expression of nod genes for node factors (lipo-chitooligosaccharides) synthesis (Corre-Hellou et al. 2007). Node factors initiate root hair curling (infection threads) and facilitate bacterial entry into roots for nodule formation, which is functional site of nitrogen fixation (Chang et al. 2017). The flavonoid signaling in rhizosphere is hindered by different soil constituents such as nitrogen and organic matter, leading to decreased nodulation and biological nitrogen fixation (Valle et al. 2020, Liu et al. 2020). With the increase of N supply in soil, exudation of flavonoids (Naringenin, hesperetin, and genistein) decreases from the roots (Liu et al. 2020).

Nitrogen accumulation in different plant parts is inversely related with C/N ratio which influence flavonoids biosynthetic pathways and reduce flavonoids content in roots (Deng et al. 2019). Nitrates destroy indole acetic acid, decreasing lectin binding site by host plant that leads to the hinderance in the formation of root hair curling (infectious thread). Without infections thread formation in roots, attachment of rhaizobia on roots

is constrained. Even if nodules formation occurs, nitrate conversion to nitrite damage nodule and cause nodule senescence. Complex formation of leghemoglobin and nitrous oxide in root nodule results in the loss of leghemoglobin oxygen carrying ability and thereby BNF process is inhibited (Dogra and Dudeja 1993). Soil organic carbon (SOC) content of 2.03% to 3.80% is required for utmost inoculated Rhizobium growth in soil (Swanepoel et al. 2011). The high amount of organic carbon in the soil decreases flavonoid signaling up to 70% which leads to 75% reduction in root nodulation.

Flavonoids are found throughout the plant kingdom and belong to a diverse group of aromatic compounds (Harborne and Williams 1998). Mainly these compounds are found in high concentrations in the epidermis of leaves and the skin of fruits and play various important roles as secondary metabolites. Flavonoids are also known as 'specialized metabolites' due to their species-specific metabolism in plants (Lattanzio et al. 2008). They are also known as 'natural biosensors'. In recent years, flavonoids have become the point of attention for scientists about abiotic stress conditions. Warren et al. (2003) anticipated that levels of kaempferol and quercetin (derivatives of flavonoids) in leaves of *Populus trichocarpa* boosted under exposure of UV-B irradiation (Pietta et al. 1998; Warren et al. 2003) related C glycosylflavones level of different rice cultivar under UV-B light. UV-B light enhanced the C glycosylflavones in a tolerant cultivar of rice but was not detected in a cultivar that was susceptible (Caasi-Lit 2005).

A variety of other environmental stresses like salinity, cold and water deficit also cause the up-regulation of flavonoids biosynthetic genes. Anthocyanin biosynthesis highly up-regulated in response to drought stress in ripening fruit (Castellarin et al. 2007). Yang et al. (2007) stated that flavonoid production upsurges under water deficit conditions in *Glycyrrhiza inflata* Batal in its suspension culture (Yang et al. 2007). Experiments of Ghanzanfar and Walid (2006) advocated the existence of anthocyanins; flavones and phenolics are connected to amplify the tolerance to salinity in sugarcane crop (Ma et al. 2014). Yuan et al. (2012) stated that under water deficit conditions, the expression of numerous flavonoids biosynthesis genes is augmented in *Scutellaria baicalensis Georigi* roots (Yuan et al. 2012). Ithal and Reddy reported flavonoids biosynthetic pathway rice genes OsAns and OsDfr, are induced by factors like drought, salinity and Abscisic acid, in 2004 (Ithal and Reddy 2004). The chief cause of

worldwide crop loss is considered as abiotic stresses which are responsible for the reduction of average yield for most crops by more than 50% (Zhu 2002). These stresses amend metabolic function in plants because of up-regulation of various genes leading to plant adaptation and the mitigation of the stress effects.

Drought stress is one of the major environmental factors that restrict peanut crop production worldwide. Plants show high level of biochemical, physiological and genetic responses to the drought stress. Various studies have shown that wild species of groundnut can tolerate various stress factors which includes two major ones which are drought and fungus(Guimarães et al. 2012) These two have greatly affected the yield and production of cultivated peanuts. In the vegetative organs and tissues of peanut the buildup of flavonoids is also affected by several biotic and abiotic stress conditions. The accumulated flavonoids not only help in adapting with surrounding environment moreover in overcoming stress conditions.(De Micco and Aronne 2012). It is apparent that abiotic stress factors effect development and subordinate metabolite production in higher plants (Shen et al. 2010).

Peanut growth was affected by water shortage dropping shoot dry weight, dry, number of nodules and dry weight as nitrogen content. However, there was an increase in root dry weight reaching a major exploratory surface. In addition, severe drought stress production of hydrogen peroxide coupled with protein and lipid damage was induced in peanut, but as a strategy there was an increase in soluble sugar and abscisic acid content to deal with with drought stress. Moreover, biochemical and physiological parameters were completely reversed when plants were rehydrated (Furlan et al. 2012). Drought stress causes a decrease in level of solute potential and an increase in soluble sugars in cells to regulate turgor at a lower water potential in nodules. Carbon metabolism, nodule permeability to oxygen and nitrogen feedback, are three main aspects regarding biological nitrogen fixation on which drought stress has a significant effect.

The low availability of nitrogen and other nutrients to plants affects their growth, defense, and physiology. For growth and optimal performance, nutrients must be provided to plants in the right amount and ratio and in a form that can be used at the right time. To meet these needs, chemical fertilizers or organic fertilizers are required and used by farmers. Nitrogen fertilizers are today an indispensable part of modern

agricultural practices and are found as the number one external input to maximize agricultural production (Dubey and Rai 1995). Recent studies indicate that a significant proportion of the human population depends on synthetic nitrogen (N) fertilizers to provide the 53 million tons of N that are harvested worldwide each year in food crops (Bindraban et al. 2015). In 2016, the global demand for nitrogen fertilizers is expected to reach 116 million tons per year, an increase of 5.2% compared to 2012 and an increase of 5.4% in North America, and the price of nitrogen fertilizers has tripled since 2005 (Yang et al. 2017). However, excessive use of N fertilizer causes hazardous effects on human health by leaching into ground water, and losses as nitrous oxide (a powerful greenhouse gas), leads to low N utilization efficiency as compared to nitrogen from biological nitrogen fixation (Bonilla and Bolanos 2009). Moreover, nitrogenous fertilizers' prices have increased by 336 percent from 2001to 2019 (Sharif et al. 2020)

About 55% of nitrogen required for growth and development is detained from biological nitrogen fixation in the peanut thZat could be enhanced (Hardarson 1993). Leguminous plants with low biological nitrogen fixation need more nitrogen as basal dose fertilizers for growth and development. The fertilizers use in Pakistan has increased 14-fold on per hectare basis, N use has increased from 20 kg to 133 kg between 1970s to 2014 (Ali et al. 2016). On the flip side, nitrogen fertilizer has negative consequences like it can affect the global nitrogen cycle and increases atmospheric nitrous oxide. There are large areas of the developing world where nitrogen fertilizers are not available or affordable due to poor infrastructure and high costs. Even in the richest countries, economic and environmental considerations dictate the search for organic alternatives, which can increase and, in some cases, replace nitrogen fertilizers (Bohlool et al. 1992). Nitrogen fertilizers also affect flavonoid exudation from plant roots that ultimately affects biological nitrogen fixation because flavonoids play an important role in symbiosis quorum sensing.

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.

As expected, the nutritional state of the soil has a huge influence on the symbiosis, as well as the independent growth and survival of both plant and rhizobacteria. Changes in the chemical and physical properties of the soil subsequently influence its biological functioning and nitrifying communities. The use of different types of nutrients is gaining popularity, especially in agriculture management. Such as the use of biochar in geographic areas where the addition of biochar to degraded soils can improve soil characteristics and therefore lead to higher crop yields. Biochar can absorb nitrogen, phosphorous, other nutrients, and N fertilizers and release them through the cation exchange process (Horel et al. 2019).

Nitrogen fixation by cowpea and purple vetch was more restricted by additions of ammonium sulphate than by any other substances used. Growth and nodulation of red clover (Hopkins and Fred 1933) and peas (Mulder 1949) was also more restricted by ammonium than by nitrate, but ammonium stimulated nodule formation and nitrate was inhibitory in lucerne (Richardson, Jordan, and Garrard 1957) and soya bean (Yoshida and Yatazawa 1967). Thus, the response to ammonium varies with the plant species and probably also with the growth rate of plant. The inhibition of nodulation by ammonium sulphate may be due to low pH levels at the root surface even though the bulk solution has a pH of 6-7. Ammonium ion rapidly enters the soil base exchange complex or is nitrified In the soil and is unlikely to be inhibitory since the free ammonium levels will be low (Sabagh et al. 2020). While contributing essential nitrogen for plant growth. in the soil the ammonium ion is released and forms a small amount of acid, lowering the pH balance of the soil,. It contains 21% nitrogen and 24% sulfur (Sugimoto et al. 2021).

Chapter 3

Materials and Methods

3.1. In Silico Analysis

3.1.1. Identification and annotation of nodulation genes in Arachis hypogaea

Initially nodulation genes (NFP, SYMRK, NUP85, NUP133, CCaMK, CYCLOPS, NSP1, NIN, ERN1, Nod, Nol) of Lotus Japonicus and Arachis Guridansis were taken from (Peng et al. 2017). To retrieve homologous FASTA sequences of these genes in NCBI (https://www.ncbi.nlm.nih.gov/) a local BLASTP algorithm search was used. As query FASTA sequences retrieved from NCBI were used to carry out Blastn searches in peanut genomic database PeanutBase (https://www.peanutbase.org/) with cutoff e-value of 0e. All gene sequences were checked in the pfam database (http://pfam.xfam.org/) for domains and to verify the reliability of results and to confirm each predicted sequences (Mistry et al. 2021) with manually identified full-length genes, half-length genes and genes having no domains in ORF and so, all the redundant sequences were removed and full-length 12 nodulation genes were selected for further insilico characterization. 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 determine the amino acid or open reading frames of *Nod* inducing genes. Chemical and bio-physical parameters of *Nod* inducing genes in *Arachis hypogaea* were predicted by protPARAM (http://expasy.org/tools/protparam.html) available at ExPASY by using primary sequences of genes (Gasteiger et al. 2003). These properties were predicted to find out 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 Phylogeny inference

The sequences of *Nod* inducing genes were retrieved from peanut base (https://www.peanutbase.org) and using MEGA 10.2.4 tool

(https://www.megasoftware.net/) multiple sequences of full gene length were aligned at default setting by using "align by muscle". An unrooted tree was also constructed by using MEGA 10.2.4 tool with neighbor joining (NJ) algorithm. To test the reliability of tree, the bootstrap replicates of 1000 with 50% cutoff values were used and then the tree was visualized.

3.1.3. Gene structure and conserved motif distribution analysis

By using online gene display server (http://gsds.gao-lab.org), structural information of *Nod* inducing genes i.e., intron/exon patterns were predicted (Guo et al. 2007). Gene structures are predicted in BED file format by using coding regions (CDS) of genes. For significant functional and conserved protein motifs prediction, the MEME (https://meme-suite.org/meme/) Program was used . This analysis was performed by adjustment of factors as: the optimum motif width: 3 residues, number of unique motifs: 10 and distribution of motifs: any number of repetitions (Bailey et al. 2015).

3.1.4. Chromosomal distribution and gene duplication events

From PeanutBase (https://www.peanutbase.org), the position information of NIG gene family and chromosomes length were acquired. Online visualization tool PhenoGram (http://visualization.ritchielab.org/phenograms/plot) was used on 20 Arachis Hypogaea chromosomes to visualize the relative distances and physical locations of the *NIG* gene family. To evaluate the tandem and segmental duplication events, divergence time and selective pressure of NIG-family genes, the synonymous (Ks) and non-synonymous (Ka) values were calculated by TB-tool software (https://github.com/CJ- Chen/TBtools) (Chen et al. 2020). The divergence time was calculated by using a formula T=Ks/2x*MYA (where x=6.56 x 10^{-9} and MYA= 10^{-6}) (He et al. 2016).

3.1.5. Subcellular localization prediction and sequence logos analysis

To predict and better comprehension, subcellular localization perform on NIG family proteins for several functions. All predicted 12 DNA FASTA sequences of Arachis Hypogaea were translated to protein sequences via online Expassy translate tool (https://web.expasy.org/translate/). Then we used online website Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) to locate their locations (Chou and

Shen 2010). Plant-mPLoc predictor allows localization of plant proteins at different 12 targets i.e., Chloroplast, Cell wall, Endoplasmic reticulum, Cytoplasm, Extracellular, Mitochondria, Golgi apparatus, Nucleus, Plasma membrane, Peroxisome, Plastid, Plasma membrane and Vacuole.

Moreover, Sequence logos analysis perform to determine the amino acids that are conserved or non-conserved among all the genes. Hence for sequence logos analysis, proteins of 12 *NIG* family genes were aligned by CLUSTALW (https://www.genome.jp/tools-bin/clustalw) (Thompson et al. 1997) and sequence logos were generated by WebLogo (https://weblogo.berkeley.edu/logo.cgi) (Crooks et al. 2004).

3.2. Wet lab work

3.2.1 Seed and soil Collection

Healthy and pathogen free seeds of peanut variet (Golden) were collected from the department of oil, seed and research at National Agriculture Research Centre (NARC), Islamabad, Pakistan. Sandy loamy soil from 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. To evaporate maximum ethanol, seeds are then spread on filter paper in surface sterilized safety cabinet. Completely dried seeds were then wrapped in aluminum foil and placed at 4^{o}C 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 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 temperature of 25-28^{o}C. After ten days' seeds started germinating and were ready to transfer to soil. Seeds with equal germination rate were transferred into pot for growth in the greenhouse.

3.2.3. Soil Analysis

Soil analysis was performed 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 and Plant Growth

The soil used for growing plants was arranged for four treatments. First two treatments contain soil media while last two treatments have 1.5% biochar. The nitrogen source is Ammonium sulfate ((NH4)2SO4) as shown in fig 3.1. All treatments are illustrated in given tables 3.1. Along with nitrogen sources other soil nutrients such as calcium (Ca), Sulfur (S), potassium (K), phosphorus (P) were also added as solution to all treatment as required per kg which are 15mg, 24mg, 18.6mg, 17mg and 14mg, respectively. Soil nutrients requirement for soil are given in table 3.2 below and their calculation are given in table 3.3. 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. After adding solutions soil was mixed in a tub for each treatment and after mixing, one kg soil was added to labelled 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 28^{o}C with 16h/8h light condition.

Table: 3.1. All Treatments; Ammonium sulfate ((NH4)2SO4) with and without Biochar are shown in given table.

Treatments	Nitrogen Source
T 1	Control
T 2	(NH4)2SO4
T 3	Biochar (1.5%)
T 4	1.5 % Biochar + (NH4)2SO4

Fig:3.1 Plants arranged in four treatments with nitrogen source Ammonium sulfate ((NH4)2SO4)

Table: 3.2. Soil nutrients calcium (Ca), Sulfur (S), potassium (K), phosphorus (P) requirements are shown in the given 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

Table: 3.3. Soil Nutrient Calculation

Treatments	T1: Control	T2: (NH4)2SO4	T3: Biochar	T4: Biochar + (NH4)2SO4
Total Soil	1kg	1 kg	0.955 kg 1 kg	0.955 kg
Biochar required (1.5%)	-	-	15g	15g
Chemical required	-	(NH4)2SO4= 70mg	-	(NH4)2SO4= 70mg

3.2.5. Water Holding Capacity

Took 3 pots for simple soil and 3 pots for soil containing 1.5% biochar for calculating water holding capacity of the soil media. I did 5 holes in each pot in the bottom of the pots and put tissue inside each of them. Soil and soil having 1.5% biochar were added into the pots having empty space of one inch above in all pots. After that, three pots having only soil were placed in one water tub and other three pots having 1.5% biochar in other water tubs. 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 equal amount of time. Weight of the pots was measured which is W1. Then soil was removed from the pots covered in aluminum foil and dried in drying oven for 4 to 5 hours at 105 C. Soil weight was measured after drying (Wa). At room temperature, pots were dried and weight was measured (Wb). Obtained 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, add 65% water holding field capacity to all pots. Then measured the water lose on the alternative day. And maintained the water lose throughout experiment as shown in table 3.4.

Table: 3.4. Measurement of Water Holding Capacity

	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

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). Tap root length was measured on the 45th day (harvesting day). Number of nodules were 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.

3.2.10. Chlorophyl Content

Total chlorophyll used for measured chlorophyl content by using a spectrometer. Three values were taken and then averaged was determined for each plant in healthy state. Leaf sample was taken directly after harvesting about 1cm square and 0.05mg. grounded with mortar and pestle. During grounding small amount of acetone and magnesium carbonate was added. The sample was collected in a small falcon tube and centrifuged for 2 minutes at 13000 rpm. Acetone was used as a control. Calculations for chlorophyll concentrations are made after the absorbances are read wavelengths. (Guo et al. 2017; Zhu 1993).

Chapter 4

Results

4.1. Identification and annotation of NIG family in Arachis hypogaea

A total of 12 *NOD* inducing genes in Arachis hypogaea were identified with Sequence homology analysis. To retrieve homologous FASTA sequences of these genes in NCBI a local BLASTP algorithm search was used. Using gene annotations and Blastn searches in peanut genomic database PeanutBase with cutoff e-value of 0e for gaining NIG family genes from peanut. All NIG family genes identified encoded proteins containing the nodulation gene function related domains and were analyzed for nodulation process according to their location on the chromosome. The ExPASY translate tool was used to deduce the amino acid or open reading frames of *Nod* inducing genes. Each candidate gene contained the conserved domain was determined and then confirmed the presence of related domains based on Pfam analysis by pfam database. Based on sequence identity with the functionally characterized NIG family, the individual names of all genes were given. The gene names of the NIG family, their accession numbers, length of the coding sequences, and characteristics of these proteins are shown in Table 4.1. The chemical and bio-physical parameters of *Nod* inducing genes in Arachis hypogaea were predicted by protPARAM available at ExPASY by using primary sequences of genes. These properties were predicted to explore important characteristics of genes e.g., coding sequence (CDS), protein length (aa), isoelectric point (pI), molecular weight (MW), aliphatic index (AI), extinction coefficients (EC) by assuming all pairs of Cys residues form cystines, grand average of hydropathicity (GRAVY) and their estimated half-life.

The full-length coding sequences of the *Nod* inducing genes ranged from 1155 bp (AhNSur13) to 1394 bp (AhNSur8) and their putative proteins contained between 384 and 471 amino acid (aa) residues, with an average of ~416 aa. The theoretical pI ranged from 6.13 to 8.08 and molecular weights ranged from 42957.07 (AhNSur10) to 52449.43 (AhNSur8). In congruence with the

features, the genome-wide studies also detected significant variation in aliphatic index, extinction coefficients and GRAVY inferring a high degree of complexity and functional diversification among the NIG family of Arachis hypogaea.

Table: 4.1. Characterization of NIG family genes in *Arachis hypogaea* (PL: protein length (No. of amino acids) ,PI: isoelectric point, Mw: Molecular weight , II: instability index, AI: Aliphatic index, EC: Extinction coefficients (assuming all pairs of Cys residues form cystines), GRAVY: Grand average of hydropathicity, Half-life: The time required for a quantity to reduce to half of its initial value).

Sr. No	*Gene ID*	*Chromosomes*	*Accession numbers*	*Nucleotide CDS (bp)*	*Length (aa)*	*PI*	*Mw*	*II*	*AI*	*EC*	*GRAVY*	*Half life (hours)*
1	AhNSur7	13	MZ170073	1314	438	6.19	48600.88	31.79	79.61	65625	-0.332	>10
2	AhNSur8	03	MZ170074	1394	471	6.31	52449.43	34.31	80.25	76750	-0.288	>10
3	AhNSur9	14	MZ170075	1170	390	7.53	43585.90	30.48	80.85	76625	-0.282	>10
4	AhNSur10	04	MZ170076	1155	384	7.54	42957.07	27.62	80.60	78115	-0.315	>10
5	AhNSur11	13	MZ170077	1359	452	8.08	50105.22	34.39	86.19	68605	-0.276	>10
6	AhNSur12	01	MZ170078	1275	424	6.64	47951.66	38.58	78.33	58385	-0.398	>10
7	AhNSur13	12	MZ170079	1158	384	6.47	43819.72	39.84	76.07	58385	-0.483	>10
8	AhNSur14	02	MZ170080	1170	390	6.24	44544.47	42.63	75.67	58385	-0.494	>10
9	AhNSur15	12	MZ170081	1245	412	6.33	46813.03	37.37	74.95	56895	-0.506	>10
10	AhNSur16	02	MZ170082	1245	412	6.13	46837.01	37.78	75.90	56895	-0.510	>10
11	AhNSur17	10	MZ170083	1245	412	6.13	46837.01	37.78	75.90	56895	-0.510	>10
12	AhNSur18	20	MZ170084	1245	412	6.13	46837.01	37.78	75.90	56895	-0.510	>10

4.2. Evolutionary analysis of NIG gene family

To assess evolutionary relationship between 12 *NOD* inducing genes under this study, sequences of all the identified genes of NIG family (*Nod* Inducing Gene family) were aligned and a phylogenetic tree was constructed using neighbor-joining method with 1000 bootstrap. As shown in Fig 4.1, the evolutionary analysis divided NIG family genes of Arachis hypogaea into three major sub-families referred to as NIG-a, NIG-b, and NIG-c which, in general appeared to represent 12 members. NIG-a has 5 members, contained *AhNSur7, AhNSur8, AhNSur9, AhNSur10* and *AhNSur11*. Furthermore, Conserved domain analysis revealed that NIG-b family was the smallest family with 3 members *AhNSur12, AhNSur13* and *AhNSur14* and clustered together in a phylogenetic tree. The sub-family NIG-c has 4 members *AhNSur15, AhNSur16, AhNSur17* and *AhNSur18* contained several functionally characterized domains required for nodulation. According to their domains, they are characterized as rhizobial infection and nodulation causing genes which is specific to nodulation and required for symbiotic relationship. Hence these genes are dependently originated which show homology and revealed evolutionary relationships of *Nod* inducing genes in NIG family. The domains of these genes are structurally similar causing rhizobial infection and nodulation which leads to biological nitrogen fixation.

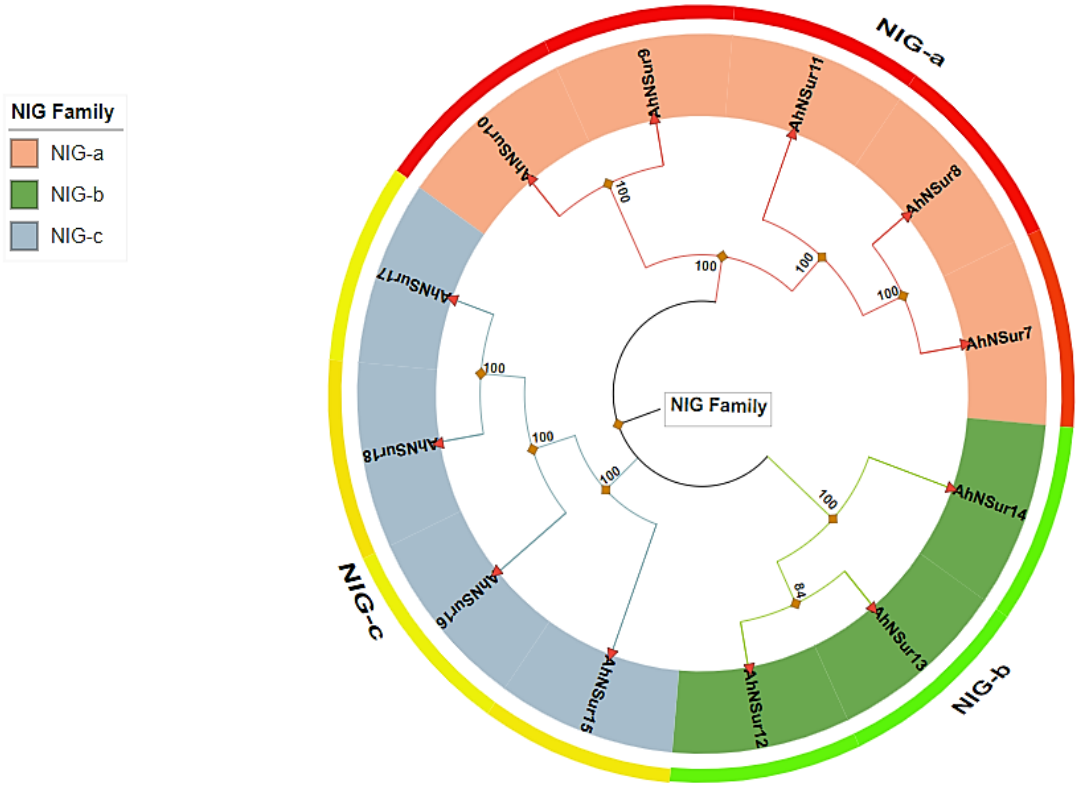

Fig:4.1. Phylogenetic tree of NIG family (*Nod* Inducing Gene family). The tree was constructed using 12 sequences with Neighbor-joining method and bootstrap values shown on the nodal branch.

4.3. Conserved motif analysis and intron/exon organization of NIG family genes

Conserved motifs of *NIG* family genes were predicted by utilizing online MEME tool. 12 genes of *NIG* family presented 10 motifs with different amino acids were found with its detailed information including name, width and best potential matches. However, the amino acid length of these motifs ranges from 15-50. Identified motifs and their schematic distribution in all members are shown in Fig 4.2. However, the sequence for each conserved motif is shown in table 4.2. The numbers of motifs were comparatively greater in *NIG*-a sub-family than *NIG*-b and *NIG*-c sub-families. Motif 1 to 6 was found in all *NIG*-a, *NIG*-b and *NIG*-c genes. The analysis suggest that motif 1-6 could be signature motifs associated with *NIG* family genes. Similarly, motif 7 was exclusively

present in all Nodulin stress up-regulating genes (except for *AhNSur14*, *AhNSur15*, *AhNSur16* and *AhNSur17)*. Motif 8 were documented for all genes of *NIG*-a and *NIG*-b families. Motif 9 was associate to all NIG family genes except *AhNSur9*, *AhNSur12* and *AhNSur13*. Motif 10 was encountered in all genes except *AhNSur9* and *AhNSur13*, which could be related with diversifications in their functions. Furthermore, we found that the conserved domain in genes of *NIG* family positioned at similar locations. The results demonstrate that all of the genes of this family are closely related as they have similar composition and might have similar function of motif which represents that the genes structures are highly conserved. The conserved motif prediction is essential to further gain understanding of The similarities and structural characteristics of among these motifs can be studied further to provide new insights.

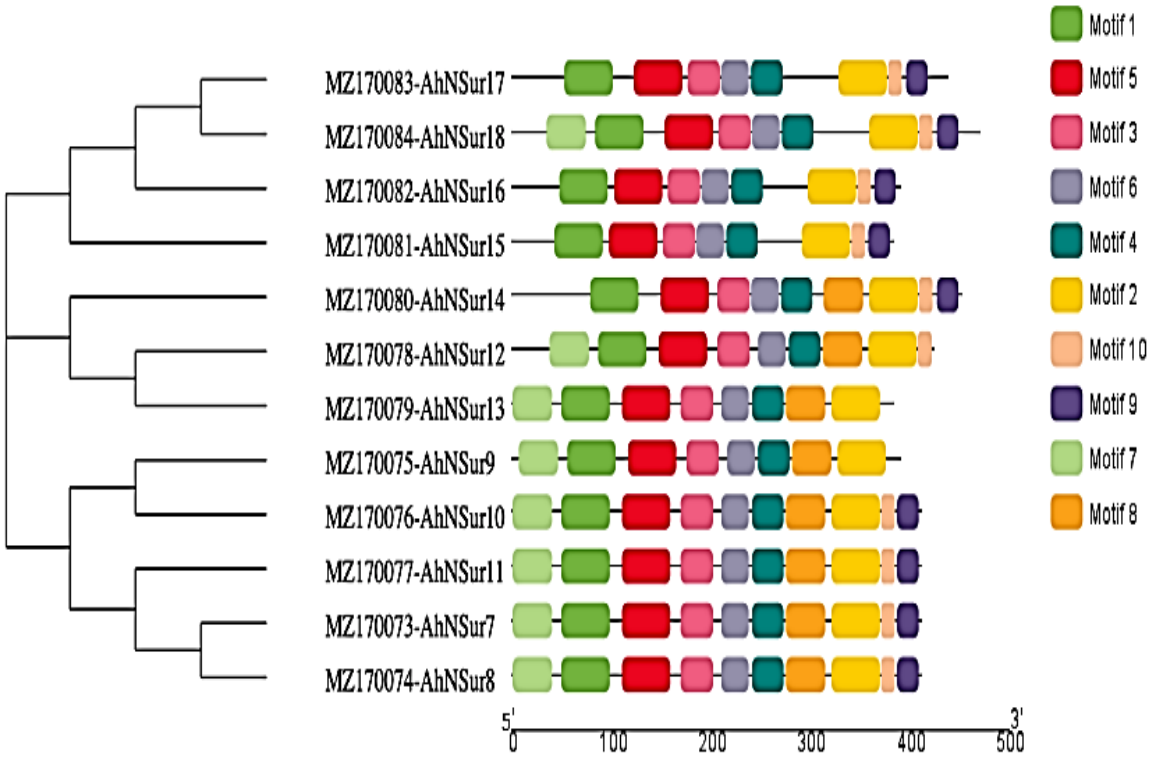

Fig: 4.2. Representation of 12 sequences for *NIG* family genes which shows the presence of 10 motifs

Table: 4.2. Motif sequence analysis showed the conserved amino acid sequences, e-values, sites and width of the sequences

Motif	*Conserved amino acid sequences*	*e-values*	*Sites*	*Width*
1.	VSNKTYFDIEFPRGHIGJKNFQAELVDEHGNSVPLYEAYLHHYFVLRYFE	3.2e-377	12	50
2.	AHVHSGIVNATLYGZDGRVJCESKPIYGTGEEAGNEKGYAVGMSGCYPKP	8.5e-359	12	50
3.	KWLJNIHVIDTRGVEDKKGCTECRCDLYNVKSED	2.5e-254	12	34
4.	QKRKLYLKYTVKWVDWDQYQVPLKFYILDVTDQ	1.5e-230	12	33
5.	QPNYGKYFKRNDGVCQGNVNSYSWGLGVDARGTSTEJPDPFRIEVGTHPE	4.2e-300	12	50
6.	DGKPLSSDYKGGJFCCEKESQCKLQKGY	2.9e-194	12	29
7.	MKFICEVVJLSFSIIVJQSSITFSRZLEGPNHIKTTTFYTK	4.2e-157	7	41
8.	TYNGSEPIHHCAVEYSINPZKTDEGHYHIKKTNIPMKKGGS	1.6e-174	7	7
9.	SETKYHTGVMGLFYLLVAEDLPH	2.5e-093	9	23
10.	GSMKIKDGENLTLEF	1.7e-052	10	15

To analyze the exon/intron structure of *NIG* family genes BED files were used, the analysis suggested that all are full length genes i.e., the domains for nodulation lie inside the CDS regions. However, there are intronic regions in all these genes and these intronic regions are upstream the CDS regions. (as shown in fig 4.3). These observations indicated that each group in NIG family, showing maximum common gene structural conservation. Furthermore, structural and functional characterization of *NIG* family genes are important to gain information about the evolution of this gene family

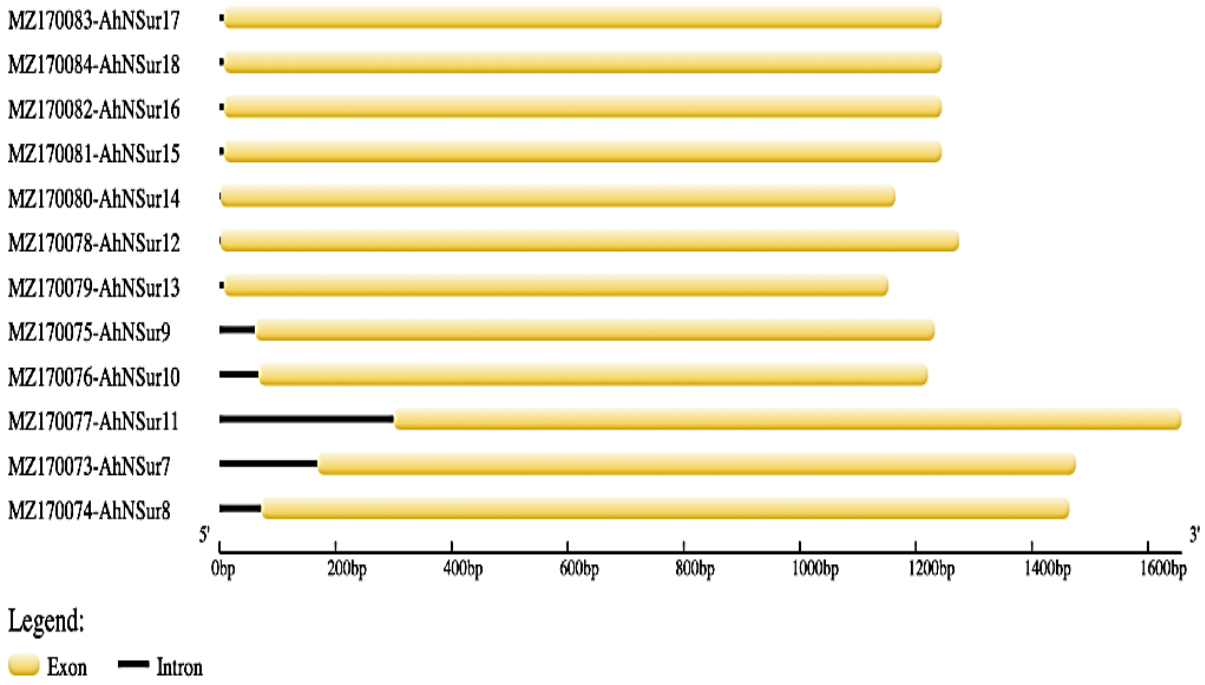

Fig: 4.3. Exon/Intron Structure of NIG family Genes

4.4. Chromosomal location and gene duplication of *NIG* genes

The genes were widely distributed throughout *Arachis hypogaea* genome. Chromosomal location of 12 *NIG* gene family were investigated on their corresponding 20 *Arachis hypogaea* chromosomes. Genes were mapped and named on chromosome according to their order (Fig 4.4).So, their distribution indicated that genes were unevenly distributed on chromosomes. Chr2, Chr12, Chr13 has higher number of *NIG* genes as compared to others i.e., two genes (*AhNSur14* and *AhNSur16*) on Chr2, (*AhNSur13* and *AhNSur15*) on Chr12 and (*AhNSur7* and *AhNSur11*) on Chr13. While there was no *NIG* gene of this study located on Chr5, Chr6, Chr7, Chr8, Chr9 Chr11, Chr15, Chr16, Chr17, Chr18 and Chr19. Among 20 chromosomes, 6 chromosomes contained maximum number of 1 gene (Chr01: *AhNSur12,* Chr03: *AhNSur8,* Chr04: *AhNSur10,* Chr10: *AhNSur17,* Chr14: *AhNSur9*, Chr10 and *AhNSur18* respectively). In plants, propably duplication mechanisms occur during gene families expansion; usually

these mechanisms involve segmental duplication, tandem duplication and whole-genome duplication (Su et al. 2020).

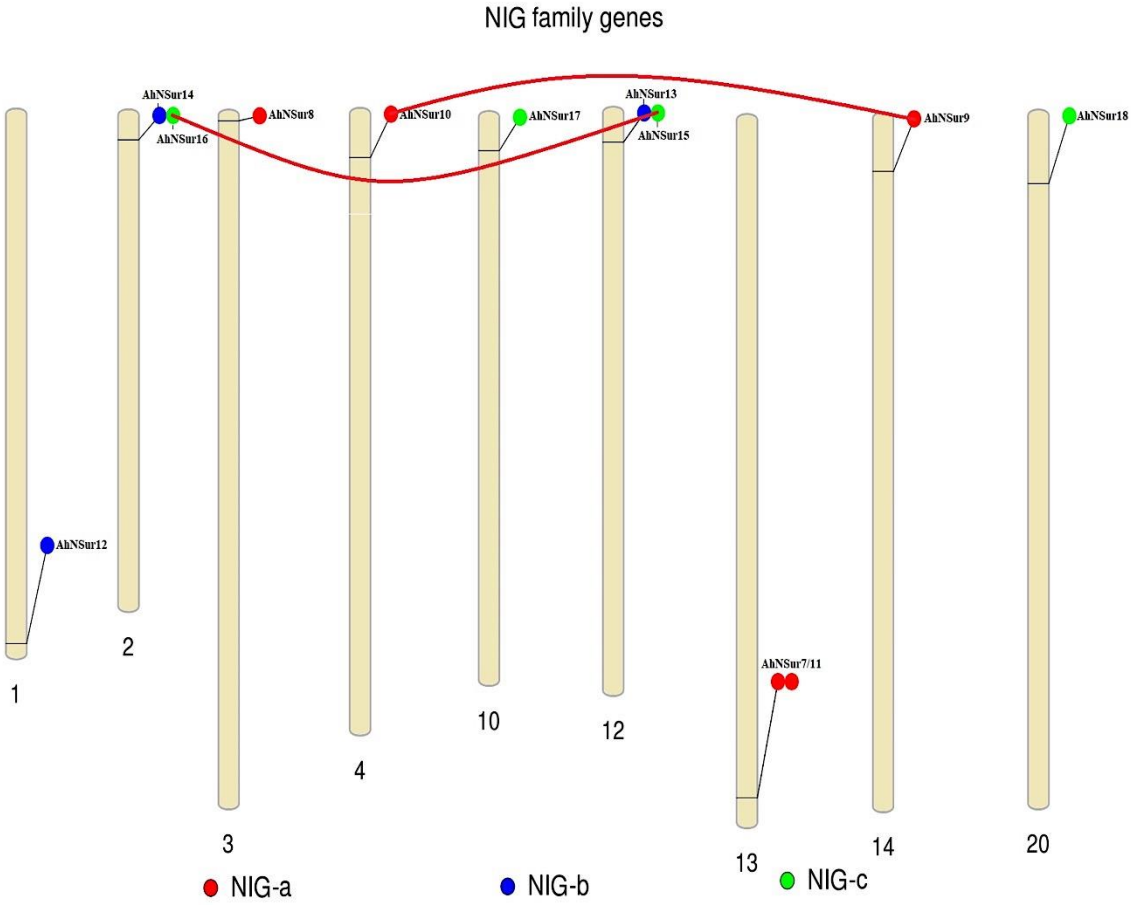

Fig: 4.4. Representation of Chromosomal location and gene duplication of *NIG* genes.

Hence to understand possible relationship between *NIG* gene family and potential gene duplication within *Arachis hypogaea* genome, analyzation was done through the duplication mechanisms during the evolution of this gene family here (as shown in table 4.3). By analyzing the sequence coverage and similarities of 12 *NIG* genes, 2 pairs of duplicated genes (*AhNSur9/AhNSur10, AhNSur15/AhNSur16*) were identified that experienced segmental duplication, as depicted in Fig 4.4. While there was no tandem and whole genome duplication event. These observations were suggested that segmental duplication contributed largely to the expansion on NIG family members in

Arachis hypogaea. Ka and Ks values for these duplicated gene pairs were calculated by using KaKs calculator and then obtained information was used to determine selective evolutionary pressure. Both gene pairs had Ka/Ks<1 which indicated purification selection and segmental duplication event of 2 gene pairs were predicted to occurred between 0.398 and 0.864 million years ago.

Table: 4.3. Duplication Mechanisms During The Evolution Of NIG Gene Family

Sr.No	*Paralogous Pairs*	*Ka*	*Ks*	*Ka_Ks*	*Duplication*	*Time (Million years ago)*
1.	AhNSur09-AhNSur10	0.0113413	0.06329949	0.17916888	SD	0.86442829
2.	AhNSur15-AhNSur16	0.00522741	0.02578523	0.20272897	SD	0.39843085

4.5. *NIG* family protein subcellular localization and sequence logos analysis

By Plant-mPLoc analysis, the protein localization predicted that all proteins of *NIG* family are located at single position. For instance, these single location proteins are present in chloroplast rather than nucleus and cell membrane. (as shown in fig 4.5(a)).

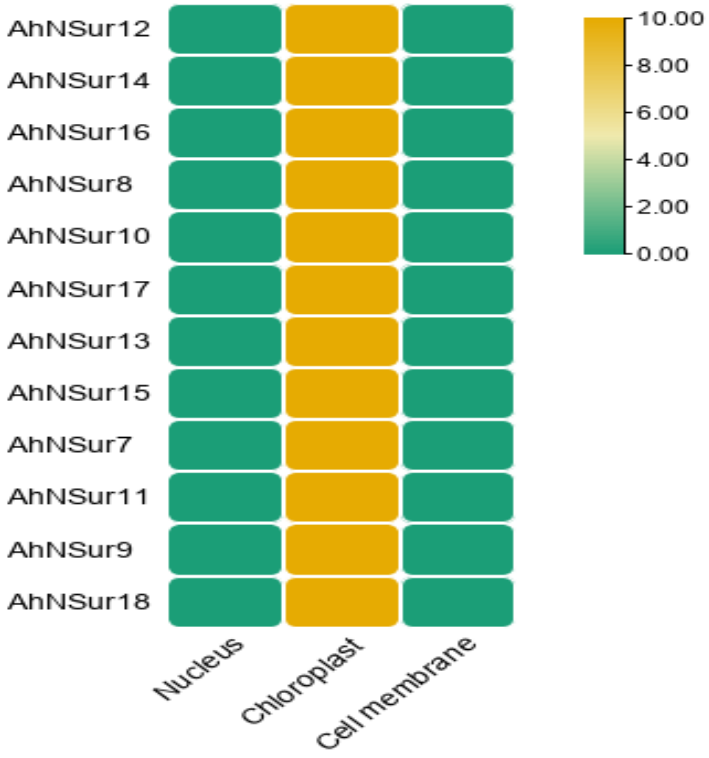

Fig: 4.5(a) Heat map of 12 NIG family genes generated with Tbtool software.

In all the 12 genes, sequence logos of *NIG* family proteins could help to evaluate and discover the pattern of protein conservation. In *Arachis hypogaea,* to determine whether the *NIG* family proteins were conserved in all the 12 genes throughout evolution sequence logos of aligned amino acid residues generated (as shown in Fig 4.5(b)). The analysis showed that the protein sequences had high level of conservation at many different positions across N to C terminal.

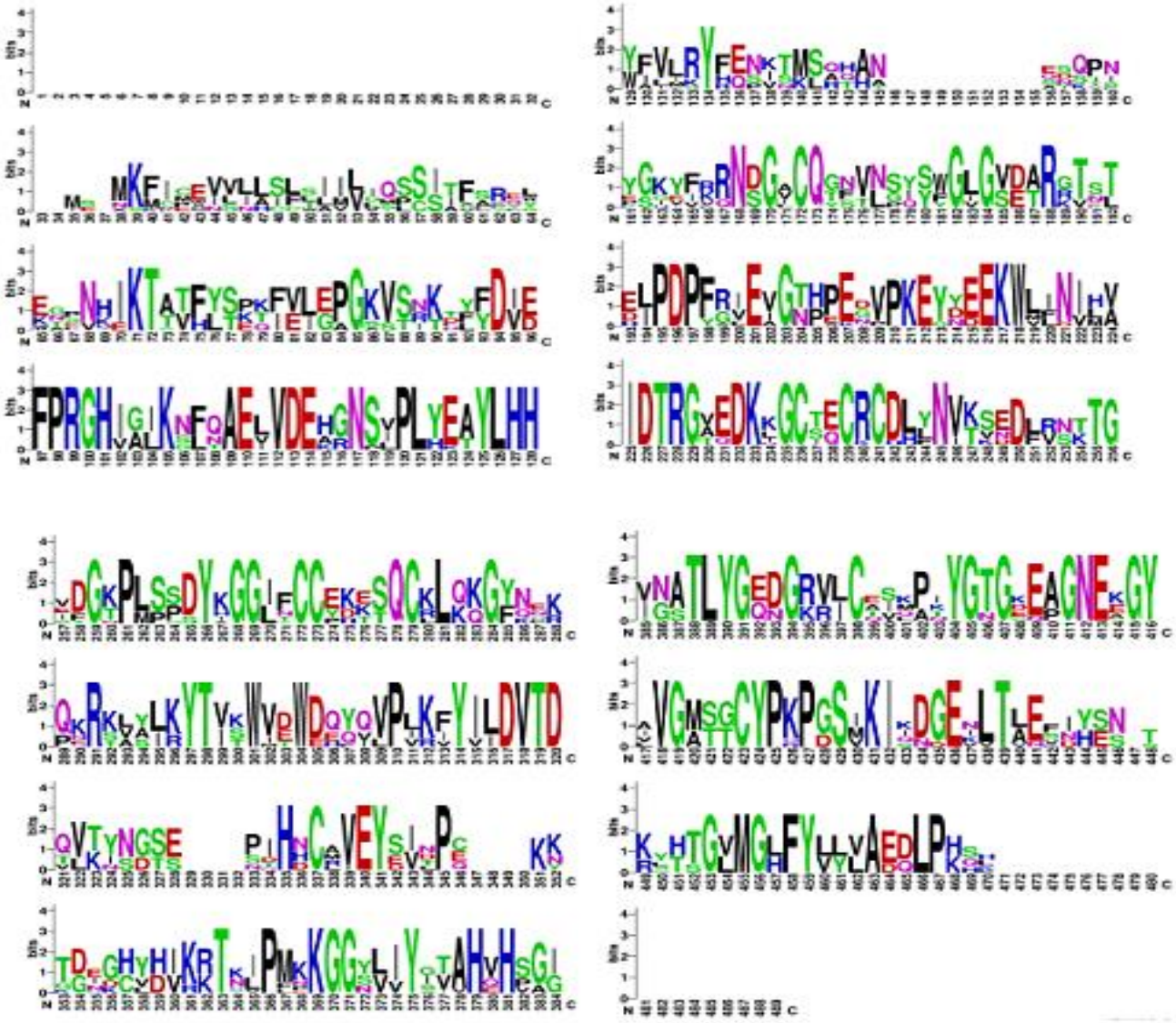

Fig : 4.5(b) Sequence logos of NIG family genes. The N-terminal and C-terminal of NIG gene domain are indicated by using 'N' and 'C'.

4.6. Phenotypic analysis and total chlorophyll content

The number of leaves and branches were counted on 14th, 21st, 28th, 35th, 42nd, and 45th days after sowing. Shoot height 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 rinsed 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.

4.6.1. Number of branches

Nitrogen sources integrated with and without biochar had no significant effect on number of branches (P = 0.54831, R^2 = 0.15613, Fig. 1). However, T3 (biochar) resulted in maximum number of branches as compared to other treatments.

4.6.2. Number of leaves

Significant effect has been observed for Leaves number upon application of nitrogen sources with and without biochar. Although, maximum number of leaves has been observed in T3 (biochar) while minimum number of leaves observed in T4 ((NH4)2SO4)+ biochar).

4.6.3. Total fresh and dry weight

Total fresh weight showed no significant effect by nitrogen sources incorporated with and without biochar (P = 0.71181, R^2 = 0.10421, respectively). While total dry weight also showed no significant effect by nitrogen sources incorporated with and without biochar (P = 0.7908, R^2 = 0.08019 , respectively).

4.6.4. Height

Nitrogen sources with and without organic amendment had no significant effect on height of the plant (P = 0.90325, R^2 =0.04465). However, T1 (control) and T3 (biochar) showed maximum and minimum effect on height.

4.6.5. Root length

Root length was significantly partial by nitrogen sources alone and with biochar. Although, T3 (biochar) and T2 ((NH4)2SO4) resulted maximum and minimum root length.

4.6.6. Nodules number

Number of nodules was significantly influenced by nitrogen sources without and with biochar. The number of nodules was higher in T1 (control) and lower in T2 ((NH4)2SO4).

4.6.7. Total chlorophyll content

Total chlorophyll concentration in plant tissue was significantly altered by nitrogen sources with and without biochar. T2 (NH4SO4) was found with maximum total plant tissue chlorophyll concentration. However, T1 (control) was recorded with lowest total chlorophyll concentration in plant tissue.

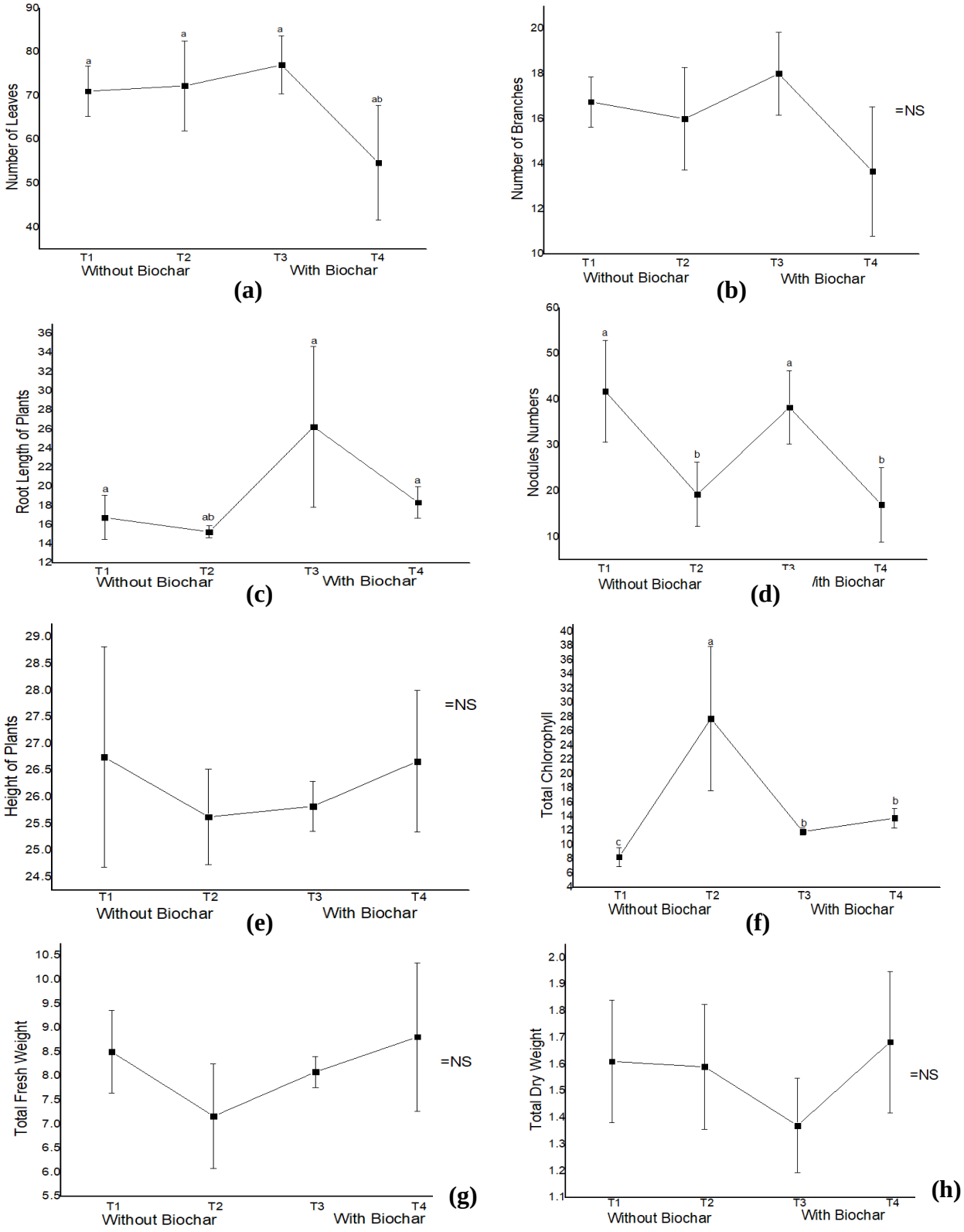

Fig:4.6 (a,b,c,d,e,f,g,h) show effect of (NH4)2SO4 and biochar application. The line bar is showing the treatment means and the error bar is showing standard error of treatment means.

Chapter 5

Discussion

Peanut is a globally important legume crop and no plant of interest comparable to Arachis hypogaea has received botanical attention to the man in recent decades. The identification and characterization of the NIG family genes have increased our understanding of nitrogen fixation and flavonoid in Arachis hypogaea. It is nutritionally important legume crop, having 25-28% protein, 48-50% oil content and they are supplier of naturally active flavonoids such as polyphenols, flavonoids and isoflavones. 5664 kcal energy per 100g of peanut kernel is produced (Jambunathan 1991). Moreover, peanut could be utilized to prevent lymphatic disorders and to boost immunity. Due to the presence of resavtrol and oligomeric procyanidins, peanut seeds are used to prevent the formation of cancer cells (Afrin 2015). Peanut oil is an outstanding cooking medium because it has high level smoking point. In addition to peanut seeds, peanut shells are of great importance they are used as fertilizers, in the feed industry and for making particle board (Arya et al. 2016). Arachis hypogaea forms symbiotic relationship with rhizobacteria in order to fix atmospheric nitrogen. The rhizobia lie in the host root nodules and in-turn fixes atmospheric nitrogen, which is then utilized by host plant to carry out several important functions (Shankar et al. 2021). Until now, bioinformatics analysis of nodulation genes in Arachis hypogaea has not been reported. Therefore, by using query sequences of other legumes, this study identified 12 *NIG* genes.

The genome wide analysis characterized these genes into three sub-families and total members in each sub-family was 3, 4 and 5. The domains identification in all 15 genes confirmed their nodulation role in Arachis hypogaea. However, in order to understand potential functions of proteins, their bio-physical properties were computed, which indicated that these genes have different roles in symbiotic pathway. The CDS ranged from 1155 bp (AhNSur13) to 1394 bp (AhNSur8) and their putative proteins contained between 384 and 471 amino acid (aa) residues, with an average of ~416

aa. The theoretical pI ranged from 6.13 to 8.08 and molecular weights ranged from 42957.07 (AhNSur10) to 52449.43 (AhNSur8). Structural analysis for *NIG* gene family showed that all the genes are full length genes some have upstream intronic regions only. Motif analysis showed 10 conserved motifs and which indicates that genes are highly conserved. The conserved motifs indicated high similarity between genes structure. The gene structure and motif identification validated the reliability of the tree classification. The genes were unevenly distributed on chromosomes, 2 pairs of duplicated genes (*AhNSur9/AhNSur10, AhNSur15/AhNSur16*) were identified that experienced segmental duplication duplication occurred between 0.398 and 0.864 MYA. These observations were suggested that segmental duplication contributed largely to the expansion on NIG family members in *Arachis hypogaea* Furthermore, the results of sub-cellular localization predicted that all proteins of *NIG* family are located at single position. The analysis showed that the protein sequences had high level of conservation at many different positions across N to C terminal.

Plant-microbial symbiosis is dependent on under-ground communications, however, essential nutrients like ammonium sulphate and biochar may influence this relationship (Shah and Smith 2020; Valle et al. 2020). Node factors initiate root hair curling (infection threads) and facilitate bacterial entry into roots for nodule formation, which is functional site of nitrogen fixation (Chang et al. 2017). As expected, the nutritional state of the soil has a huge influence on the symbiosis, as well as the independent growth and survival of both plant and rhizobacteria. Changes in the chemical and physical properties of the soil subsequently influence its biological functioning and nitrifying communities. So, the use of different types of nutrients is gaining popularity, especially in agriculture management. Such as the use of biochar in geographic areas where the addition of biochar to degraded soils can improve soil characteristics and therefore lead to higher crop yields. Biochar can absorb nitrogen, phosphorous, other nutrients, and N fertilizers and release them through the cation exchange process (Horel et al. 2019). In the present study, application of ammonium sulphate with and without biochar found no significance results like in biomass production which suggests ammonium sulphate applied at 30kg per hectare is not environment friendly approach. For economical and impactful outcomes in agriculture sector as compared to currently available fertilizers, analysis conform there is a need of appropriate fertilizer which is not only economical

but also minimize the side effects of leaching, soil degradation and green house gas effects. Thus, local farmers and fertilizer industries will gain profitable benefits from the product and might influence the foreign exchange earnings when exported.

Chapter 6

Conclusion

In the current study, identification and comprehensive genome-wide characterization of NIG family genes was systematically conducted in *Arachis hypogaea*, a versatile agronomically important plant species. The Present work represents classification, chromosomal locations, and conserved motifs of 12 NIG family genes in *Arachis hypogaea* . In addition, these genes will provide insight in their intron/exon positions, as well as in terms of their functional characterization. Segmental duplication contributed largely to the expansion on NIG family members in *Arachis hypogaea* which were clustered into three sub-families. Biophysical properties indicated that NIG family genes localized to single compartment, which suggested that the proteins are associated with specific functions. The determination of key genes strongly indicated their potential role in response to biotic and abiotic stresses which might be implicated in growth and development, detoxification, and secondary metabolite transport. In the present study, application of ammonium sulphate with and without biochar found no significance results like in biomass production which suggests ammonium sulphate applied at 30kg per hectare is not environment friendly approach. And suggests appropriate conditions which is not only economical but also minimize the side effects of leaching, soil degradation and green house gas effects. The results provide useful information for further study related to structure, function and developmental research in peanut and other valuable plants.

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