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The Role of the Auxin Biosynthesis and Signaling in Soybean Root Nodule Development

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THE ROLE OF THE AUXIN BIOSYNTHESIS AND SIGNALING IN SOYBEAN ROOT NODULE DEVELOPMENT

BY

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THESIS ACCEPTANCE PAGE

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This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This dissertation is dedicated to my parents, Gulnaz Bozkus and Bilal Bozkus.

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ABSTRACT

THE ROLE OF THE AUXIN BIOSYNTHESIS AND SIGNALING IN SOYBEAN ROOT NODULE DEVELOPMENT

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Nitrogen(N) is one of the most important plant nutrients for plant growth and yield, however, its abundance in the soil is not sufficient for profitable crop production. The use of chemical fertilizers helps address soil N deficiency in agriculture. However, due to the environmental pollution resulting from excessive use of fertilizers, alternative forms of N for agriculture are a necessity. Leguminous plants such as soybean (*Glycine max*) form a symbiotic association with N-fixing rhizobia to meet their N demands. Legume-rhizobia symbiosis results in the formation of unique structures called nodules where rhizobia convert atmospheric nitrogen into plant usable forms, thus reducing the need for chemical fertilizer application. The plant hormone auxin plays a crucial role in determining the number of nodules and their rate of maturity in soybean. A better understanding of what roles auxin plays in regulating nodule number and nitrogen fixation would help devise strategies to enhance or optimize N supply from nodules of soybeans and other legumes.

Indole-3-acetic acid (IAA) is the most abundant natural form of auxin produced by plants. The conversion of tryptophan to IAA through the two-step IPA pathway is the main source of auxin in Arabidopsis. Auxin action is mediated by a group of F-box proteins belonging to the TIR/AFB family that act as receptors. In this study, we evaluated the role of auxin biosynthesis and signaling in soybean root, nodule development, and nitrogen fixation

using yucasin and PEO-IAA, chemicals that inhibit auxin biosynthesis and signaling respectively. The suppression of auxin biosynthesis with the use of yucasin in soybean roots led to a significant increase in root length and to a significant reduction in the expression of the *IAA9*, an auxin-responsive marker gene. Moreover, the suppression of auxin biosynthesis in soybean roots led to a significant increase in total nodule and mature nodule number at 21 days post rhizobia inoculation (dpi). The expression levels of *FWL1*, *ENOD2*, *ENOD40*, and *NSP1* showed an increasing trend but the expression level of these markers was not significantly increased. The application of yucasin did not significantly affect nodule nitrogenase activity per plant and nodule. Pod number, seed number, seed weights, and seed protein concentration were used as a grain yield measurement in soybean and results showed that yucasin treatment did not affect grain yield.

The suppression of auxin signaling with the use of PEO-IAA in soybean root led to increase in root length and a reduction in lateral root density. The expression level of auxin-responsive genes showed variable expression levels. Moreover, the application of PEO-IAA in soybean root increased the total number of nodules and the mature number of nodules at 10 and 20 μM at 21 dpi. The expression of *ENOD2*, *ENOD40*, *NIN*, *FWL1*, and *NSP1* had a consistent trend of increased marker gene expression at 10μM and 20μM PEO-IAA. There was a significant increase in the expression of *FWL1*, *ENOD2*, and *NIN* at 10μM PEO-IAA treatment. Moreover, the application of PEO-IAA did not affect any of the grain yield-related traits evaluated.

Overall, our results confirmed that auxin biosynthesis and signaling play an important role in soybean root and nodule development, and that manipulation of auxin biosynthesis and

signaling could be used to optimize nodule numbers and potentially nitrogen fixation and grain yield.

Key words: Auxin, Auxin biosynthesis, Auxin signaling, soybean, rhizobia

CHAPTER 1

1. INTRODUCTION

1.1. NITROGEN IN AGRICULTURE

1.1.1. IMPORTANCE OF NITROGEN

Nitrogen is a key component of many important biomolecules such as DNA, RNA (HOWARD AND REES 1996), and amino acids (WAGNER 2011). Therefore, it is the second most important input for crop production after water. Nitrogen is crucial for crop production also because it is a major component of chlorophyll which has a significant role in photosynthesis (May,2000). In addition, Nitrogen is a component of energy-transfer compounds, such as ATP (adenosine triphosphate). ATP allows cells to sustain and use the energy released in metabolism. Plants comprise around 4 % nitrogen in their above-ground tissues; this amount is higher than other nutrients (KANT 2018).

1.1.2. AVAILABILITY OF NITROGEN

Plants obtain nitrogen mainly from the soil after it becomes available through mineral decomposition. Nonetheless, the decomposition of minerals is a slow process and is not able to fulfill the nitrogen requirement for crop production. Thus, modern agriculture depends mainly on artificial nitrogen fertilizer to obtain maximum crop yield. Nitrogen fertilizer application for agricultural production in the world has risen over 8-fold since 1961 (Lu and Tian, 2016). According to FAO statistics, from 2015 to 2019, nitrogen fertilizer consumption and demand for N have been increasing. However, chemical fertilizers contribute to around 50% of fossil fuel usage in agriculture (CANFIELD *et al.* 2010). The use of chemical fertilizer is not sustainable as only less than half of the chemical fertilizer applied is used by plants while the rest is lost by leaching to the environment

(WESTHOFF 2009). This loss of nitrogen causes extra costs for farmers and threatens the environment because of air and water pollution and its negative impacts on soil health and biodiversity. Therefore, the over-use of fertilizer results in several environmental and ecological problems, such as air pollution, soil acidification, and crop yield reduction (MCCAULEY *et al.* 2009). Thus, there is a strong need to reduce our reliance on chemical nitrogen fertilizers and instead optimize alternative nitrogen inputs.

1.2. BIOLOGICAL NITROGEN FIXATION

Biological nitrogen fixation (BNF) is an alternate source for agricultural nitrogen needs. Biological nitrogen fixation is the process where atmospheric nitrogen is converted into ammonia, a useable form of nitrogen, by a group of prokaryotic organisms, called diazotrophs (DE BRUIJN 2016). These prokaryotes contribute to plant nitrogen needs in symbiotic, associative, or free-living forms (DIXON AND KAHN 2004). In free-living nitrogen fixation, microorganisms in the soil fix atmosphere nitrogen which is utilized by plants and other organisms in the environment. These bacteria live in soil and can survive without the direct influence of plant roots. *Azotobacter*, *Clostridium*, *Rhodospirilum,* and various cyanobacteria are free-living nitrogen-fixing (NF) prokaryotes. The associative form where N-fixing bacteria are in closer contact with plants (present in the root surface or inside the root) is prevalent in several crop plant species (e.g. corn, rice, vegetable crops). The symbiotic form where N-fixing bacteria are in a tightly coordinated association with host plants provides the maximum nutrient benefit to the plant. Legume plants and Rhizobia bacteria are the most common examples of symbiotic nitrogen fixation. The symbiosis leads to specialized organs termed nodules where nitrogen fixation occurs.

Nonetheless, the biological nitrogen fixation process requires the nitrogenase enzyme to convert nitrogen into usable ammonia (HOWARD AND REES 1996). The reaction during BNF occurs as follows:

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2\,NH_3 + H_2 + 16ADP + 16\,Pi$$

Nitrogenase catalyzes the reduction of protons to hydrogen and the reduction of diverse alternative substrates such as acetylene, azide, or cyanide. Encouraging biological nitrogen fixation in agricultural systems could decrease the need for chemical fertilizers, resulting in ecological sustainability and economic benefits.

1.3. LEGUME-RHIZOBIA SYMBIOSIS

Legume crops play a critical role in agriculture as they contribute to more than 25% of world food production (HELENIUS AND STODDARD 2007). Moreover, the legume-rhizobia symbiosis produces annually around 200 million tons of nitrogen (PEOPLES *et al.* 2009). Soybean (*Glycine max* L. Merr.) is one of the most important legume crops for vegetable oil, its high protein, and nutrition, and its seeds are used as animal feed and human food. (CONSULTATION 2011; MUTAVA *et al.* 2015). Soybean as leguminous crops provides a special benefit in sustainable agriculture by providing nitrogen in a natural way (STAGNARI *et al.* 2017) as it has developed a symbiotic relationship with nitrogen-fixing bacteria called rhizobia (WANG *et al.* 2012). As a result of a symbiotic relationship, a unique structure known as a root nodule is formed in leguminous plants. Bacteroids inside these nodules offer reduced nitrogen to the host plant and in return, obtain energy and carbon from the host plant. High volumes of ATP and oxygen reductant are necessary to encounter the demands of the nitrogenase enzyme; however, in the meantime, nitrogenase is oxygen-sensitive (WANG *et al.* 2012). Leghemoglobin that produces the pink color to effective

nodules binds oxygen and transfers it to the bacterial electron transport chain, and ATP synthesis, resulting in reduced concertation of free oxygen in the nodule. Carbon demand and the necessity of microaerobic conditions contribute to the high sensitivity of the nitrogen fixation process to environmental factors. Thus, development of biotechnological and genetic improvements to biological nitrogen fixation demands a better understanding of nodule development.

1.4. ROOT NODULE SYMBIOSIS

Root nodule symbiosis is initiated as a result of precise interaction between legumes and rhizobia. Nodule development can be categorized into two main biological processes: bacterial infection and nodule organogenesis (OLDROYD AND DOWNIE 2008). Legumes release phenolic flavonoids to the rhizosphere and these flavonoids attract the bacteria to the root, which leads to activation of *nod* genes and secretion of nod factors (NFS) by rhizobia (LIU AND MURRAY 2016). NFS activates nodule organogenesis and leads to cellular changes related to bacterial infection. The primary target of rhizobia is the root hair of the host plant to start the infection. Root hair tip elongation and deformation occur when bacteria attach to the root hair (Figure 1.1-1). This process is called root hair curling and the formation of an infection thread (IT) (OLDROYD AND DOWNIE 2008).

The infection thread expands into the primordia cells and the branched infection thread releases the bacteria into the host cytoplasm. Bacteria are surrounded by a plant-derived membrane named the peribacteroid membrane, resulting in structures known as symbiosomes. The bacteria continue to divide inside the symbiosome and differentiate into bacteroids that fix the atmospheric nitrogen (NAP AND BISSELING 1990; FERGUSON *et al.* 2010).

There are two major types of legume nodules, determinate and indeterminate primarily based on the persistence of the nodule meristem, a group of mitotically active cells that contribute to nodule formation and growth.

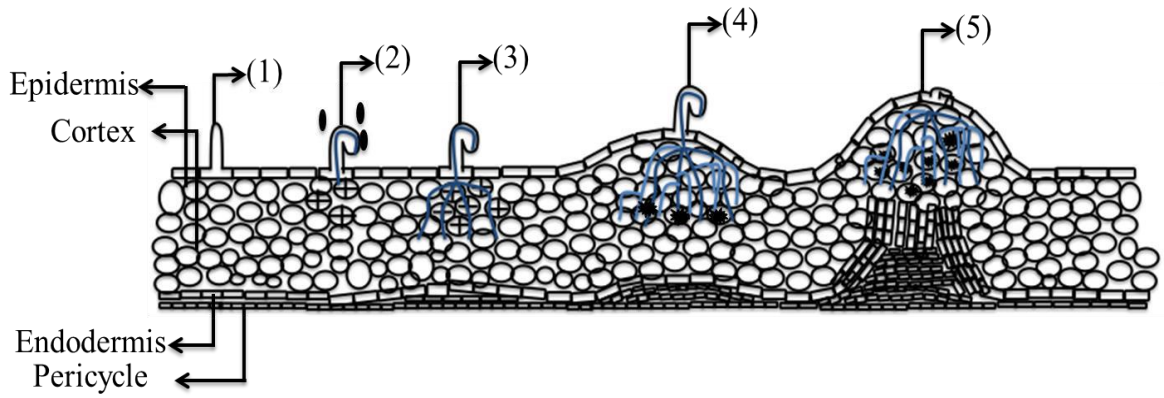

Figure 1.1-1 Stages of nodule organogenesis in determinate nodule type.Secretion of flavonoid compounds by the root hair. (2) Perception of flavonoids by rhizobia bacteria. (3) Growth and branching of infection thread into cortex cells. (4) Cell division continues in the outer cortex and pericycle cells. (5) Nodule continues to grow by elongation and differentiation (FERGUSON et al. 2010).

1.4.1. INDETERMINATE NODULES

During the initiation of indeterminate nodules, the primary cell division occurs in the inner cortex through anticlinal division followed by cell division in the pericycle, resulting in nodule primordia formation (FERGUSON *et al.* 2010). The cell division in the nodule primordia is permanent and is restricted to the meristematic zone upon nodule maturation. This continuous renewal of meristematic tissue leads to an indeterminate nodule with an elongated oblong structure with multiple functional zones. The nodule meristem in Zone I consists of constantly dividing cells; Zone II is referred to as the infection zone; Zone III is known as the nitrogen fixation zone; Zone IV is the senescence zone; and Zone V is the

saprophytic zone (GAGE 2004; FERGUSON *et al.* 2010). All these zones in the central tissue are covered at the periphery by the nodule parenchyma tissue, with vascular bundles traversing it (BOND 1948; NEWCOMB 1976). Mature nodules contain a heterogeneous population of nitrogen-fixing bacteria due to continuous cell division. This results in a spatial gradient of developmental phases, providing them an elongated stricture, and indeterminate nodules have a less branched vascular system (FERGUSON *et al.* 2010). Legumes such as *Pisum* (pea), *Medicago* (alfalfa), *Trifolium* (clover), and *Vicia* (vetch) produce indeterminate nodules (BOND 1948; NEWCOMB 1976) (Figure 1.1-2).

1.4.2. DETERMINATE NODULES

Determinate nodules lack a persistent meristem; maturation consists primarily of cell elongation rather than division, resulting in spherical shape for mature nodules (TURGEON AND BAUER 1982). Moreover, during determinate nodule development, initial cell division occurs anticlinal in the outer cortex cell and subsequently in the root pericycle and its adjacent inner cortex(FERGUSON *et al.* 2010). The cells dividing in the root outer cortex differentiate into the central tissue, though the dividing pericycle and inner cortex result in the parenchyma tissue which surrounds the central zone (HIRSCH 1992). The nodule vascular tissue traverses the parenchyma tissue in the periphery of the mature nodule. Inside the infection zone, the bacteria convert the atmospheric nitrogen and transfer the assimilated nutrient via the uninfected cells and through the nodule vasculature. Mature determinate nodules contain a homogenous population of N-fixing bacteroids because infected cells are differentiated synchronously. Determinate nodules are mostly found in tropical and sub-tropical species like soybean (*Glycine max*), bean (*Phaseolus vulgaris*) (Figure 1.1-2)

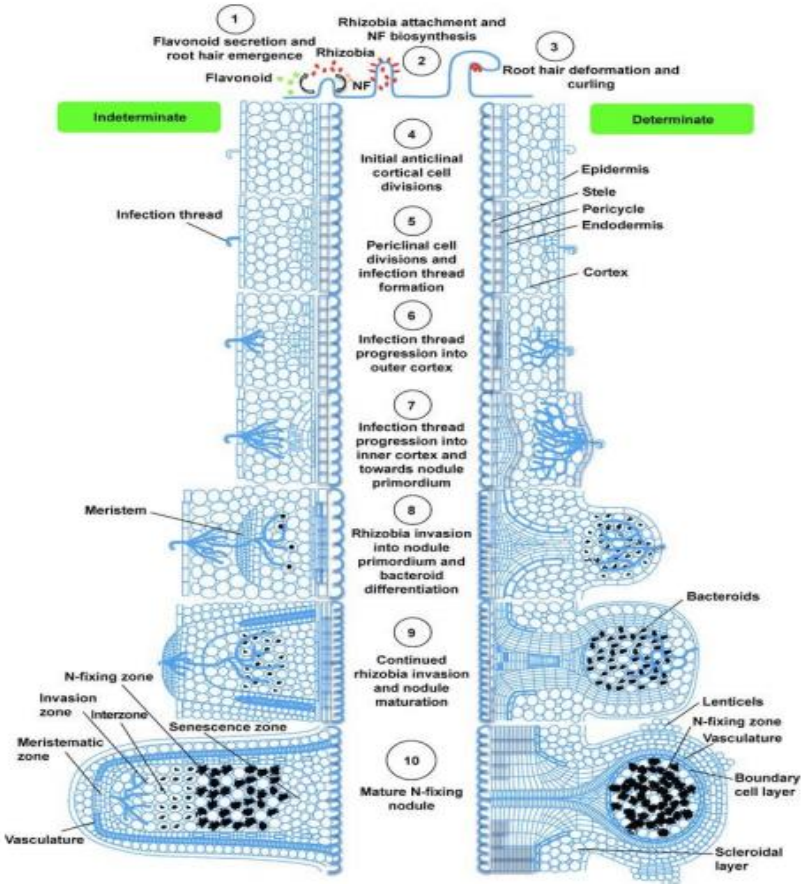

Figure 1.1-2 Two main types of root nodules. The distinct stages of nodule development in both nodule types, indeterminate nodule shown in the left and determinate nodule illustrated in right (FERGUSON et al. 2010).

1.5. AUXIN AND NODULE DEVELOPMENT

Most of the information related to auxin functions and their mechanisms underlying is based on information from the model plant species *Arabidopsis thaliana.* Although the

importance of auxin as a growth hormone was identified from the time the early days of plant science, the precise functions of auxin in plant developmental stages were only recently demonstrated because of genetic mutants in biosynthesis, transport, and signaling pathways of auxin (CHENG *et al.* 2006; ABEL AND THEOLOGIS 2010). The most common form of auxin existing in a plant is indole-3-acetic acid, although other auxins like indole butyric acid (IBA) and phenyl acetic acid (PAA) shas been detected in plants. Aside from the naturally synthesized auxin molecules, there are synthetically derived auxin molecules like naphthalene acetic acid (NAA), and 2,4-dichloro phenoxy acetic acid (2, 4-D). Auxin plays an important role in different plant developmental processes including embryo development, cell proliferation, vasculature, and leaf and flower development (CHENG *et al.* 2006; TAO *et al.* 2008). Auxin plays a crucial role in rhizobial infection and nodule development in legumes and it has four main functions during nodulation: rhizobia infection, formation of nodule primordia, cell cycle control within the nodule, and nodule vascular tissue differentiation (KONDOROSI *et al.* 2005; WANG *et al.* 2019).

In indeterminate nodules (e.g., produced by *M. truncatula*, *Medicago sativa*, *Pisum sativum*, and *Vicia sativa*), cell divisions occur in the inner cortex and pericycle while in determinate nodules cell divisions are restricted to the middle (e.g., *Lotus japonicus*) or outer (e.g., *Glycine max*) cortex. Several studies showed that Nod factors affect local distribution and concertation of auxin. The auxin-responsive *GH3* promoter-*β*-glucuronidase transcriptional fusion (GH3:GUS) showed strong expression in the dividing outer cortical cells at the site of nodule primordium formation in determinate nodule (*Lotus japonicus)* and up-regulation in auxin transport was detected in the root after inoculation with Nod factors (PACIOS-BRAS *et al.* 2003; KONDOROSI *et al.* 2005; WANG *et al.* 2019).

(MATHESIUS *et al.* 1998) showed the accumulation of auxin at the sites of indeterminate nodule initiation (before nodule primordia formation) by using the auxin-inducible marker (GH3:GUS). Thus, increased auxin output in the site of nodule development results in the cell division required for nodule organogenesis.

Auxin plays an important role in the differentiation of vascular bundles during nodule vascular development. Nodule vascular tissue forms in the nodule periphery, in the parenchyma tissue. Auxin response markers are expressed in the nodule vascular tissues of both indeterminate and determinate nodules, suggesting a role for auxin in nodule vascular development.

Flavonoids which are produced by plants play a role as auxin transport inhibitors (PEER AND MURPHY 2007). The inhibition of auxin transport, mediated by flavonoids, increases auxin accumulation, which is important during indeterminate nodule development (WASSON *et al.* 2006) However, flavonoid-mediated auxin transport inhibition is not essential for determinate nodule development (SUBRAMANIAN *et al.* 2007). The role of auxin regulation in the development of determinate and indeterminate nodules is likely to differ based on both studies. In soybean nodule primordia, Turner *et al.*, 2013 showed DR5(auxin-inducible marker) is expressed during the early cell divisions forming the nodule primordium in the root cortex. In mature determinate nodules, the auxin response is mainly limited to the nodule parenchyma in the periphery of the infection zone, specifically in the nodule vasculature (SUZAKI *et al.* 2013; TURNER *et al.* 2013). In indeterminate nodules such as in Medicago, auxin response is observed at the earlier phase of cortical cell division similar to that in determinate nodules such as in soybean, but the auxin response persists in the nodule apex throughout nodule development. Auxin response

observed in the nodule parenchyma, specifically in the vasculature tissue is similar to what is observed in determinate nodules (MATHESIUS 2001; MATHESIUS 2008). Thus, a spatiotemporal auxin response pattern is essential for appropriate nodule development. Loss and gain of function approach involving auxin response molecular components revealed their role in nodule development. For example, overexpression of miR160 which suppresses the expression of repressor AUXIN RESPONSE FACTORs ARF10/16/17 led to reduced nodule formation (TURNER *et al.* 2013; NIZAMPATNAM *et al.* 2015). Enhanced auxin sensitivity in nodule primordium results in reduced nodule formation. Moreover, when the level of miRNA160 was suppressed, the formation of emerging nodules was increased but the maturation of nodules was a delay, indicating that high miRNA160 levels are necessary to regulate auxin output during nodule maturation. Additionally, when the *ENOD40* promoter marker was used to express miRNA160 ectopically, the number of emerging nodules was increased, however, there was no change in the number of mature nodules. Therefore, the activity of miRNA160 might be essential in the nodule parenchyma and may not be crucial in the development of the infection zone during maturation.

Auxin biosynthesis in roots and root hairs contributes to rhizobial infection and nodule organogenesis in soybean. The *GmYUC2a, which is an important regulator of auxin biosynthesis modulating* nodulation for the YUCCA(YUC) gene family of soybean, is highly expressed in soybean nodules, it is responsive *to* rhizobial infection, and its promoter exhibited strong activity in maturing nodules (WANG *et al.* 2019). Additionally, GmYUC2a-mediated local auxin biosynthesis contributes to rhizobial infection and nodule organogenesis, including nodule senescence, in soybean nodules. (WANG *et al.* 2019).

1.6. AUXIN BIOSYNTHESIS

Auxin is an essential hormone in every aspect of plant growth and development (ZHAO 2010). Indole-3-acetic acid (IAA), the most important natural source of auxin produced by plants, is generated from two different pathways, called the tryptophan- (Trp) dependent and the tryptophan-independent pathways (ZHAO 2010; ZHAO 2012). IAA can also be generated through the conversion of conjugated hydrolytic cleavage of IAA-amino acids, IAA-sugar, and IAA-methyl ester (ZHAO 2010). The conversion of tryptophan to IAA through the two-step IPA pathway is the main source of auxin in Arabidopsis (ZHAO 2012). It has been clearly shown that Trp-dependent auxin biosynthesis is crucial for embryogenesis, seedling growth, flower development, vascular pattern formation, and other plants' developmental process (CHENG *et al.* 2006; TAO *et al.* 2008). The IPA pathway, a two-step pathway, includes transamination of tryptophan by the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA1) to produce indole pyruvic acid (IPA) (STEPANOVA *et al.* 2008; ZHAO 2010). Later, IPA undergoes oxidative decarboxylation catalyzed by the YUCCA (YUC) family of flavin monooxygenases to produce IAA (CHENG *et al.* 2006; ZHAO 2010). Aside from metabolites, there were other metabolites like indole 3-acetonitrile and indole 3-acetamide, which were known as intermediates in the auxin biosynthesis pathway, however, these metabolites may not be the main contributor to IAA biosynthesis. However, the two-step auxin biosynthesis pathway is highly conserved throughout plants and is essential for the plant developmental process (ZHAO 2010). YUCCA (YUC) flavin monooxygenase was first identified key auxin biosynthesis enzyme as overexpression of YUC in Arabidopsis leads to overproduction of auxin and it catalyzes a rate-limiting step in a Trp-dependent auxin biosynthesis pathway (ZHAO *et al.* 2001). In Arabidopsis, there are 11 YUC genes and

members of the YUC gene family have over-lapping functions during plant development (CHENG *et al.* 2006; CHENG *et al.* 2007). Inactivation of a single YUC gene in Arabidopsis does not lead to obvious developmental defects, but simultaneous disruption of several YUC genes causes defects in embryogenesis, seedling growth, flower development, and vascular pattern formation(CHENG *et al.* 2006; CHENG *et al.* 2007; ZHAO 2012). Thus, YUC genes play essential roles in auxin biosynthesis and plant development in not only Arabidopsis but also in other plant species as well (ZHAO 2012). Auxin biosynthesis in Arabidopsis has been shown to mainly occur in the young leaf primordia tissue, from where it is transported to other tissues for proper plant development (ZHAO 2014). However, genetic studies have been showed genes involved in auxin biosynthesis were highly enriched in the root apical meristem(LJUNG *et al.* 2005). Understanding the mechanism of any cell-specific expression of auxin biosynthesis genes will help us to understand genetic action of local auxin biosynthesis on auxin activity during nodule development. In legumes, Trp-derived IAA production was discovered through labelling assays that showed a possibility of the IPA pathway in this plant family, though it has been not clearly indicated (QUITTENDEN *et al.* 2009). Moreover, transcriptomics data in multiple legumes at distinctive developmental stages, involving nodule development, offers an insight into the tissue-specific expression of auxin biosynthesis genes. Thus, understanding the roles of auxin biosynthesis-related genes in soybean root nodule development could provide information revealing their role in overall auxin activity.

1.7. AUXIN TRANSPORT AND SIGNALING

Primarily, free auxin mainly remains in two forms which are called protonated (IAAH) and unprotonated (IAA-) (SAŃKO-SAWCZENKO *et al.* 2019). Due to the acidic nature of auxin,

it exists in an unprotonated or charged anionic form (IAA-). IAA- type is too polar to diffuse thus they need the help of different auxin transport proteins to exit the cell. PIN and ABCB family are auxin transport efflux carrier proteins and AUX1/LAX and PIN-LIKES proteins are auxin influx carrier proteins to help auxin exit and enter the cell correspondingly that provide cell to cell auxin flow (TEALE *et al.* 2006; HAN *et al.* 2009).

Essentially, the major mechanisms by which changes in auxin levels are converted into cellular responses is through the changes in transcription regulation. Many genes change their expression quickly in response to exogenous auxin levels (PAPONOV *et al.* 2008; LEYSER 2018). Auxin signaling generates cell responses to the various auxin levels which are occurred by a combination of auxin metabolism and transport. The transcription of auxin is regulated via an elegantly short signal transduction pathway called the TIR1/AFB family of auxin receptors (LJUNG 2013). In brief, auxin plays a role as molecular glue bringing together F-box proteins of the TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFB) family and members of the Aux/IAA transcriptional repressor family (LEYSER 2002; TAN *et al.* 2007). TIR1/AFBs (TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) that are F-box proteins. The F-box itself is a motif at the N-terminal end of F-box proteins that interact with Skp1, which also interacts with a dimer of Cullin and RBX1 to form the ubiquitin-protein ligase complex, SCF^{TIR1}. TIR1/AFBs bind IAA, forming a co-receptor complex with Aux/IAA repressor proteins, which regulate negatively auxin signaling (LEYSER 2018) (Figure 1.2). At low IAA levels, the Aux/IAA proteins and the co-repressor TPL can bind to and repress auxin response factors (ARFs) which are a group of transcription factors and regulate auxin-responsive genes. At high IAA levels, however, the formation of the (TIR1/AFB)-

IAA-(Aux/IAA) co-receptor complex targets the Aux/IAA proteins for degradation via the 26S proteasome (HAYASHI 2012) (Figure 1.2). As a result of this, the ARFs are free to bind to genes including auxin response elements (TGTCTC) in their promoters to activate or repress transcription. Thus, changes in auxin levels are converted into changes in Aux/IAA levels.

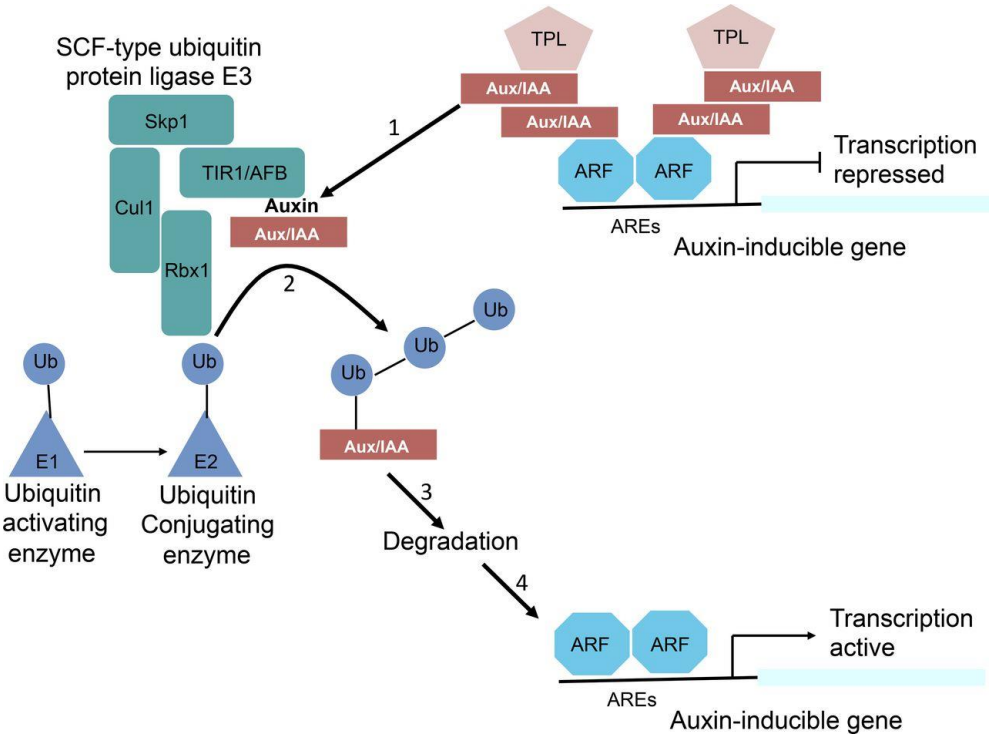

Figure 1.1-3 The main pathway auxin regulation and transcription. Auxin-inducible genes have auxin response elements (AREs) in their promoters bound by dimers of the Auxin Response Factor (ARF). Gene expression is suppressed by the recruitment of Aux/IAA transcriptional repressors interacting with the ARFs. Aux/IAAs recruit co-repressor (TPL). The steps in the auxin signaling pathway are indicated by arrows. (1). Auxin acts play a role as molecular glue bringing together Aux/IAAs and F-box proteins of the TIR1/AFB family. (2) F-box proteins are part of an SCF-type E3 ubiquitin ligase complex which transfers activated ubiquitin (Ub) from an E1/E2 enzyme. (3) Polyubiquitination of the Aux/IAAs leading to their degradation. (4) This releases repressors at ARE-including promoters.

1.8. OBJECTIVE OF THIS STUDY

Auxin plays a key role in plant development, including the development of nodules. Based on the previous studies, auxin transport inhibitors, flavonoids, increases the auxin accumulation that is crucial at the site of nodule initiation during indeterminate nodule development. However, flavonoid-mediated auxin inhibition is not crucial for determinate nodule development. Auxin response markers are expressed in the nodule vascular tissues of both determinate and indeterminate nodules, indicating a possible role of auxin and the change in the auxin response during nodule vasculature development. However, neither of these observations address the detection of high auxin accumulation at the site of determinate nodule initiation. There is one possible hypothesis that local auxin production at the nodule initial cells or in the dividing outer cortex tissue might function with coordinated auxin transport. The importance of auxin biosynthesis in primary root development and lateral root organogenesis and on cell division, differentiation, and elongation in organ development are well-reviewed. However, the role of auxin biosynthesis in nodule development still remains unclear, especially its role in nodule initiation and maturation. The objectives of this research were to evaluate the role of auxin biosynthesis and signaling in soybean root, nodule development, and nitrogen fixation. The results are expected to help reveal better information on how auxin biosynthesis and signaling affect nodule development in soybean.

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CHAPTER 2

2. THE ROLE OF THE AUXIN BIOSYNTHESIS IN SOYBEAN ROOT AND NODULE DEVELOPMENT

2.1 ABSTRACT

Soybean forms a symbiotic association with the nitrogen-fixing soil bacterium *Bradyrhizobium japonicum.* This symbiosis leads to secondary root organs called nodules where biological nitrogen fixation occurs. However, root nodules meet only 60-70% of the total nitrogen needs in soybean. Therefore, strategies to increase nodule numbers and nitrogen fixation are required. The plant hormone auxin regulates many aspects of nodule development. In soybean, lower auxin sensitivity leads to more nodules while higher auxin sensitivity leads to fewer nodules, but the role of auxin biosynthesis is not known. The major IAA biosynthesis pathway in plants is the tryptophan-dependent IPA pathway where tryptophan (Trp) is converted to IPA by TAA aminotransferases followed by conversion of IPA to IAA by YUCCA (YUC) enzyme. Yucasin is an IAA biosynthesis inhibitor that targets the YUCCA enzyme and can significantly reduce IAA biosynthesis. In this study, we evaluated if and what concentration of yucasin can reduce auxin levels in soybean and evaluated the effect on nodule development. Based on root growth, IAA quantification, and quantitative reverse transcription PCR, we show that 20 μM yucasin consistently reduces auxin levels in soybean roots. Moreover, soybean roots treated with 20 μM yucasin had a significant increase in mature nodule number. However, the application of yucasin did not significantly affect nitrogenase activity per plant and nodule or affect soybean grain yield. Overall, our results suggest that although yucasin treatment reduced auxin

biosynthesis and led to the significantly increased number of mature nodules, this did not translate to increased nitrogen fixation and grain yield.

2.2 INTRODUCTION

Most leguminous plants produce symbiotic root nodules through interactions with nitrogen-fixing soil bacteria called rhizobia. Nodule development can be mainly classified into three main temporally overlapping phases: (i) host-symbiont signal exchange for compatibility which primarily appears in the root hair and epidermal cells; (ii) nodule organogenesis and rhizobial colonization in the root cortex which involves several intrinsic plant hormonal and developmental pathways; and (iii) biological nitrogen fixation in mature nodules which involves bacterial differentiation, activation of plant nitrogen metabolism pathways, and plant-symbiont nutrient exchange. Genetic and functional genomic studies have identified several genes responsible for these different activities.

There are two distinct types of legume nodules: indeterminate and determinate (HIRSCH 1992). Indeterminate nodules are oblong and are categorized by the presence of a persistent nodule meristem analogous to lateral roots (LRs). In addition, indeterminate nodules arise from inner cortical cell layers. However, determinate nodules are spherical and lack a persistent nodule meristem and they arise from outer cortical cell layers. Plant hormones play an important role in the development of both these types of nodules (OLDROYD AND DOWNIE 2008; FERGUSON *et al.* 2010). Auxin appears to be involved in the development of both these types of nodules and plays a key role in both root hair and cortical responses. To illustrate, in soybean and *M. truncatula*, auxin perception and signaling mechanisms regulate rhizobial infection and root hair responses (BREAKSPEAR *et al.* 2014; CAI *et al.* 2017). Auxin response gene expression is observed during nodule initiation but there is

relatively low auxin output. Increased auxin output usually inhibits nodule formation in both nodule types (SUZAKI *et al.* 2013; TURNER *et al.* 2013). Moreover, precisely regulated auxin metabolism and signaling specify domains of auxin output, particularly during determinate nodule development. Thus, studies have revealed that precise spatio-temporal regulation of auxin output can be crucial for proper nodule development (BREAKSPEAR *et al.* 2014; NIZAMPATNAM *et al.* 2015). Different mechanisms including miRNA regulation influence auxin signaling during the infection and organogenesis stages in determinate and indeterminate nodules (BREAKSPEAR *et al.* 2014). Moreover, some auxin-associated genes are induced in response to rhizobium inoculation but the roles of just a few of these genes are known during nodule development.

Auxins belong to a class of essential plant hormones that coordinate almost every aspect of plant growth and development (OVERVOORDE *et al.* 2010). In roots, the most well-characterized auxin-associated phenotypes, depending on auxin level, is the length of epidermal-derived root hairs, primary root length, and lateral root density (OVERVOORDE *et al.* 2010). The proper output of auxin signaling relies on the interplay of transport, biosynthesis, conjugation, and signaling. In addition to being synthesized in the shoots, auxin is also synthesized in the roots as many of the genes involved in the synthesis of auxin are expressed in roots and that root-generated auxin contributes to the maintenance of the gradients and maxima required for normal root development. (YAMADA *et al.* 2009; OVERVOORDE *et al.* 2010).

Indole-3-acetic acid (IAA) is the key form of auxin in many plant species. The primary biosynthetic pathway for IAA appears to be the tryptophan-dependent indole-3-pyruvic acid (IPA) pathway in most dicot plants (ZHAO 2012). In this two-step pathway, tryptophan

(Trp) is converted to IPA by the TAA aminotransferase family of enzymes; Subsequently, IPA is converted to IAA by the YUCCA family of flavin monooxygenase enzymes (ZHAO *et al.* 2001). YUCCA (YUC) flavin monooxygenase was first identified key auxin biosynthesis enzyme as overexpression of YUC in Arabidopsis leads to overproduction of auxin and it catalyzes a rate-limiting step in a Trp-dependent auxin biosynthesis pathway (Zhao *et al.* 2001). Inactivation of one YUC gene in Arabidopsis does not lead to obvious developmental defects, but simultaneous disruption of several YUC genes causes defects in embryogenesis, seedling growth, flower development, and vascular pattern formation (Cheng *et al.* 2006; Cheng *et al.* 2007; Zhao 2012). Thus, YUC genes play essential roles in auxin biosynthesis and plant development in not only Arabidopsis but also in other plants.

To determine the role of auxin biosynthesis in soybean root and nodule development, different concentration of yucasin was used as a potent inhibitor of YUC (NISHIMURA *et al.* 2014). We evaluated root length, lateral root density (LRD), LR count(LR), IAA levels, and IAA-response marker gene expression in soybean root to reveal the effect of yucasin as an inhibitor of auxin biosynthesis. To evaluate the impact on nodule development, nodule count, acetylene reduction assay (ARA) for nitrogenase activity, and gene expression analysis were performed.

2.3 MATERIALS AND METHODS

2.3.1 PLANT MATERIAL

Soybean cultivar William 82 (*Glycine max* cvWilliam82) was used for the experiments. The seeds were surface sterilized using 10% Clorox for two minutes followed by 70% ethanol for two minutes. Sterilized seeds were washed with distilled water to remove the residues of Clorox and ethanol before sowing. Seeds were sown in a 4" plastic pot (Catalogue no: 14335600) filled with the autoclaved potting mixture (vermiculite and perlite in the ratio of 1:3). Seeds were regularly watered with Hoagland solution (Appendix A. Table 1). The seedlings were grown in a growth chamber using the following growth conditions: 16 hours of daylight and 8 hours of night, 50% humidity with 25 ºC during the daytime, and 20 ºC during the nighttime.

2.3.2 YUCASIN TREATMENT

To determine the effect of auxin biosynthesis on root growth and soybean nodulation, yucasin treatments were applied in the growth chamber and greenhouse. In the growth chamber, the plants were treated with different concentrations of yucasin 0, 10, and 20 μM prepared as described in (Nishimura et al. 2014). The plants were watered alternatively between yucasin mixed with N- PNS and with N- PNS. For root growth assays, wild-type soybean plants were treated with the corresponding concentration of yucasin at 3 and 7 days after sowing seeds. Plants were harvested at 10 days to measure root length, lateral root count, lateral root density, gene expression assays, and auxin level measurement. The normality of the data was determined using the Shapiro Wilk test and the statistical significance was determined using t-test in R version 3.3.0. As for studying the effect of nodulation with yucasin treatment, the plants were inoculated with *B. japonicum* at 3 days

after sowing seeds, the next day (1dpi) they were treated with the corresponding yucasin solution and again at 5, 9, and 13 dpi.

In the greenhouse, plants were inoculated with *B. japonicum* at 7 days after sowing seed and two days later, plants were treated with 0 and 20 μM of yucasin. The treatments were continued once a week for 6 weeks. The plants were watered alternatively between yucasin mixed with N- PNS and with N- PNS. Nine plants were harvested at 6 weeks after inoculation, in the V_2 stage, to measure nitrogen fixation rate (ARA). In the R_8 stage, 18 plants were harvested to measure chlorophyll content index, grain yield (average of seed number, seed weight), seed nitrogen content, and protein quantification.

2.3.3 IAA HORMONE MEASUREMENT

To measure hormone level in soybean roots treated with yucasin at 0μM, 10μM, 20μM was harvested at 10 days and sent to the Proteomics & Metabolomics Facility at the Center for the Biotechnology/ University of Nebraska, Lincoln. LC-MS was used for hormone extraction(GROVER *et al.* 2020). After extraction, the samples were reconstituted in 100 μL, then diluted by 2 and an injection at 10 μL was used as final volume.

2.3.4 NODULATION ASSAY

Bradyrhizobium japonicum, USDA110 culture was used to inoculate plants for nodulation assays. *B. japonicum* was grown in Vincent's rich media (Appendix H) supplemented with chloramphenicol (20 μg/mL) at 30 ºC on the shaker at 200 rpm for 5-7 days (O. D 600 < 0.5). At the time of inoculation, the culture was centrifuged at 3500xg for 10 min at 4 ºC and the pellet was resuspended to a final concentration of OD600nm=0.08 in nitrogen-free plant nutrient solution (N- PNS) (Appendix B. Table A2). Each plant was inoculated with 25 mL of this culture at 3 days after sowing seeds. Plants were harvested after 14 and 21-

days post-inoculation (dpi) for nodulation assay, microscopy, gene expression, and ARA. The nodules were counted at 14-21 dpi and emerging nodules were categorized to be a bump on the root surface and mature nodules are classified as a protruded structure on the root surface for nodulation assays. The nodule data was obtained from three biological replications. The data was analyzed using R studio.

2.3.5 ACETYLENE REDUCTION ASSAY

To measure nitrogenase activity of the root nodules, acetylene reduction assay (ARA) was used. The whole root with nodules was taken and transferred into glass sample tubes (volume 50 ml) sealed with rubber cork (one per seedling). Acetylene (10% of the volume of the tube) was injected and incubated for 24 hours. After 24 hours of incubation, 1 ml of air sample from each glass tube was drawn and injected into separate glass vials. Gas samples in vials and ethylene standards ranging from 10% to 0.000001% were measured using GC-MS. Sample peak areas were fitted to a calibration curve and the ethylene production was normalized to per plant and per nodule.

2.3.6 RNA EXTRACTION

The harvested plant tissues were weighed and approximately 500mg of plant tissue was used for RNA extraction TRI reagent. The whole tissues were ground using liquid nitrogen in a pre-chilled pestle and mortal until a fine powder of tissues is obtained. Approximately 5 ml of TRI reagent was added to the mixture and mixed completely. The mixture was centrifuged at 5000 RPM for 10 minutes at 4°C to remove tissue debris. The supernatant was collected in a fresh tube and 1/5 volume of Chloroform was added and incubated at room temperature for 5 minutes. The mixture was centrifuged at 5000 RPM for 15 minutes at 4°C. the clear supernatant was collected, and the step was repeated until a clear aqueous

layer is obtained. To precipitate the RNA, an equal volume of isopropanol (~2.5 ml) was added and incubated at room temperature for 10 minutes. The mixture was centrifuged at 5000 RPM for 10 minutes at 4°C and the supernatant was discarded. To the pellet added 5ml of 75% ethanol and centrifuged at 5000 RPM at 4°C for 5 minutes to remove residual salt and contaminants. The supernatant was removed after centrifugation and the pellet was dissolved in DEPC (Diethyl pyrocarbonate) treated water. The samples were quantified using NanoDrop ND-1000 spectrophotometer and the integrity of RNA was verified using agarose gel electrophoresis.

2.3.7 DNASE TREATMENT

DNase treatment of the total RNA was done using Turbo DNA-free kit (Catalogue No: AM1907). One µL turbo DNase enzyme and 0.1 volume 10x turbo DNase buffer were added to the RNA and mixed gently. The mixture was incubated at 37 °C for 30 min. DNase inactivation reagent (typically 0.1 volume) was added and further incubated for 5 min at room temperature. The tube was flicked 2–3 times during the incubation period to redisperse the DNase inactivation reagent. The mixture was centrifuged at 10,000 ×g for 1.5 min and the RNA was transferred to a fresh tube. The DNase-treated RNA was stored at -80 °C until further use.

2.3.8 CDNA SYNTHESIS

The complementary strand for the total RNA was synthesized using Maxima First-strand cDNA Synthesis Kit for RT-qPCR. Total RNA of about 3 µg was used as a template and added a combination of 4µl of 5x reaction mix and 2µl of maxima enzyme in a 200µl PCR tube. the final volume was made up to 20 µl with DEPC treated water. The thermal cycler program was set for incubation of 10 minutes at 25 ˚C, 30 minutes at 50 ˚C,

and 85 ˚C for 5 minutes. the final concentration of cDNA samples was 150 ng/ul and stored at -20 ˚C.

2.3.9 REVERSE TRANSCRIPTION- QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR)

To determine the expression levels of mRNA in the cDNA synthesized from the above-mentioned protocol using the ABI, quant studio 3 qPCR system. The reaction is set up in a 20µL final reaction volume with 10µL of 2X SYBR premix (Catalogue# 639676, Clontech, CA), 1µL of cDNA as template, 0.4µL of each 10µM forward and reverse primers, 0.4µL of 50X ROX reference dye and made up the rest of the volume with DEPC water. The reactions were setup in a 96 well plate and using the thermal cycle of 95°C for 10 sec, then 40 Cycles of amplification at 95°C for 5 secs, 55 - 62 °C for 20 sec during which the fluorescence emission from each well was collected through FAM/SYBR GREEN 1 filter.

2.3.10 PROTEIN CONCENTRATION MEASUREMENT

Pierce Coomassie Plus (Bradford) Assay Kit (Catalog # 22236) was employed to measure total protein concentration in soybean seeds. All soybean pods were harvested, and 3 seeds were taken from each plant when pods were matured. The seeds were ground, and 10 mg seed powder was used for this experiment. Powder mixed with 500 µL 1XPBS buffer, and then supernatant from each sample was transferred into new tubes. Diluted Albumin Standards (BSA) were prepared to range from 2 mg/mL to 0.025 mg/mL and standard microplate protocol was followed.

2.3.11 SEED NITROGEN CONTENT MEASUREMENT

Soybean pods were harvested when plants reached the maturation stage, and 3 seeds were taken from each plant. The seeds were ground, and 3 mg seed powder was used and saved for this analysis. Samples were analyzed at the SDSU laboratory.

2.4 RESULTS

2.4.1 EFFECT OF YUCASIN ON SOYBEAN ROOT DEVELOPMENT

To determine the effect of auxin biosynthesis in soybean root development, IAA production via the IPA pathway controlled by YUCCA gene was inhibited using the inhibitor yucasin (5-(4-chlorophenyl)-4H-1,2,4-triazole-3-thiol) (NISHIMURA *et al.* 2014). Wild-type seedlings treated with (10μM, 20μM of yucasin) showed a significant increase in root length and the number of lateral roots but they did not show any significant change in lateral root density in a dose-dependent manner (Figure 2.4-1 A, B & C). This suggested that yucasin concentration likely reduced the level of auxin. IAA hormone levels in soybean roots treated with yucasin were measured for different types of hormones which are a form of IAA or act as an intermediate during the auxin degradation pathway such as IAA, Methyl IAA (indole-3-acetic acid methyl ester), and IAA-Asp (Indole-3-acetyl-aspartate). The IAA-Asp level was significantly reduced at 20 μM of yucasin (Figure 2.4-1 D).

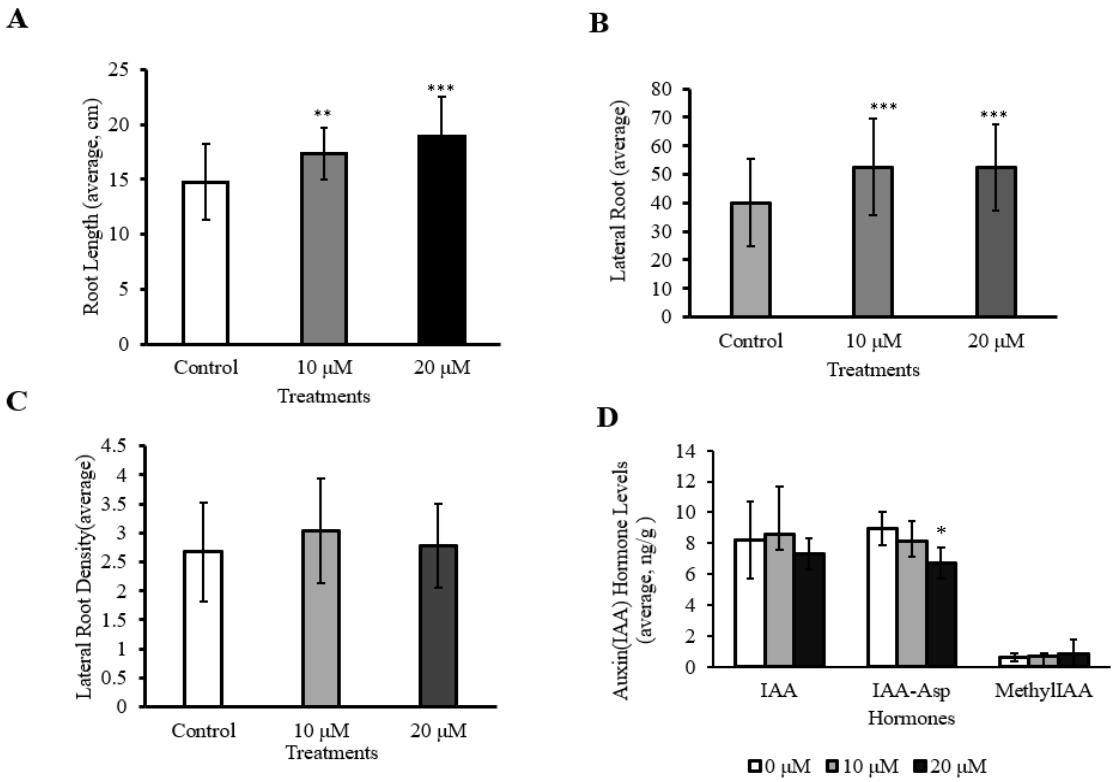

Figure 2.4-1:Effect of yucasin on soybean root development. (A, B &C) Root length (A) Lateral root count (B) and lateral root density (C; the number of lateral roots/ cm of root length) of wild-type soybean plants treated with yucasin at 0µM, 10µM and 20µM concentration for 10 days. Data are shown in A, B, and C are an average of 27 plants from three biological replicates; Error bars indicate SD, Student's t-test. ** -P<0.01, *** -P<0.001. (D) IAA, IAA-Asp, and Methyl IAA hormone measurement of wild-type soybean plants treated with yucasin at 0µM, 10µM, and 20µM. Data shown in D is an average of 3 plants from three biological replicates; Error bars indicate SD, Student's t-test. * - P<0.05.

When the auxin output marker gene expression was analyzed in yucasin treated soybean roots, seedlings treated with 10µM and 20 µM yucasin had a consistent trend of reduced marker gene expression except for *GH3*; especially, there was a significant reduction in the

expression of *IAA9* in 10μM yucasin treatment (Figure 2.4-2). Thus, 20 μM yucasin treatment might consistently reduce auxin levels in soybean roots.

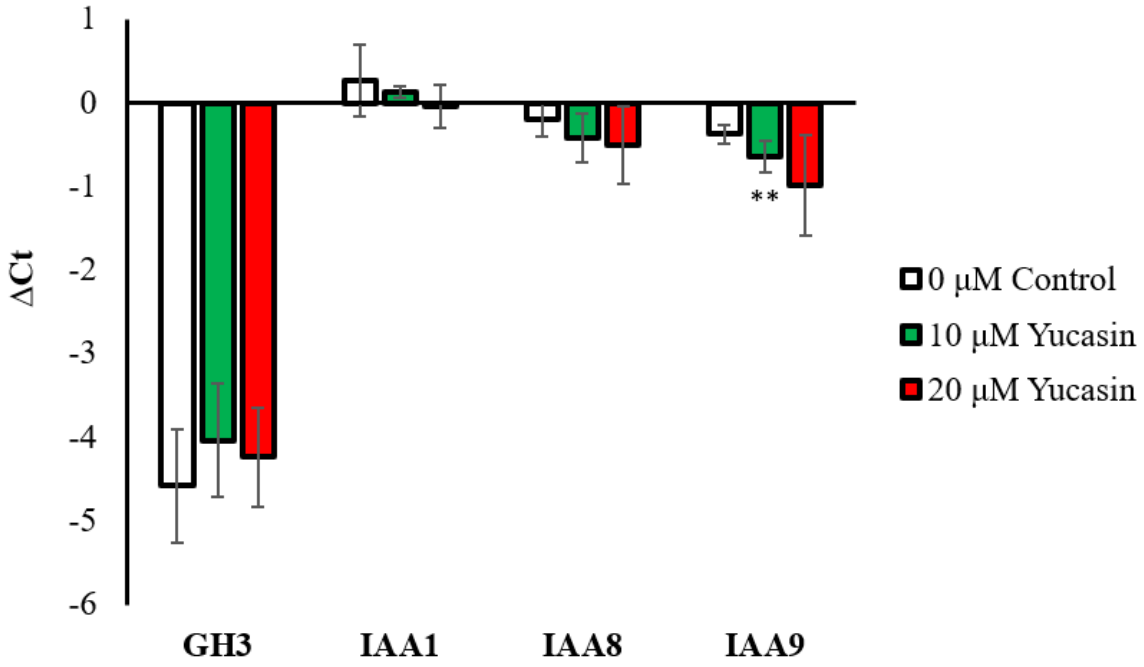

Figure 2.4-2: Expression patterns of marker genes GH3, IAA1, IAA8, and IAA9 at 10 days. Expression levels of auxin response marker genes, in wild-type soybean plant roots treated with yucasin at 0μM, 10μM, and 20μM concentrations for 10 days. Delta Ct values (normalized to Actin) are plotted on the y-axis and genes at the x-axis. Data shown are the average of 3 biological replicates**;** Error bars indicate SD, Student's t-test ** -P<0.01.

2.4.2 EFFECT OF YUCASIN ON SOYBEAN NODULE DEVELOPMENT

To evaluate the effect of auxin biosynthesis in soybean nodule development, wild-type soybean plants were treated with different concentrations of yucasin (10μM, 20μM) along with mock solvent control (0μM). Total nodule number, emerging and mature nodule number (EN and MN) were measured as a proxy of nodule initiation and maturation at different harvest days, 14 dpi and 21 dpi. The number of emerging and mature nodule numbers was not significantly altered in plants treated with yucasin and harvested at 14 dpi (Figure 2.4-3 A). However, plants treated with yucasin displayed a significant increase in the number of MN at 21 dpi (Figure 2.4.2 B). The total number of nodules was significantly increased in wild-type soybean plants treated with 10μM, 20μM of yucasin at 21 dpi, but not in plants harvested at 14 dpi (Figure 2.4-3 C).

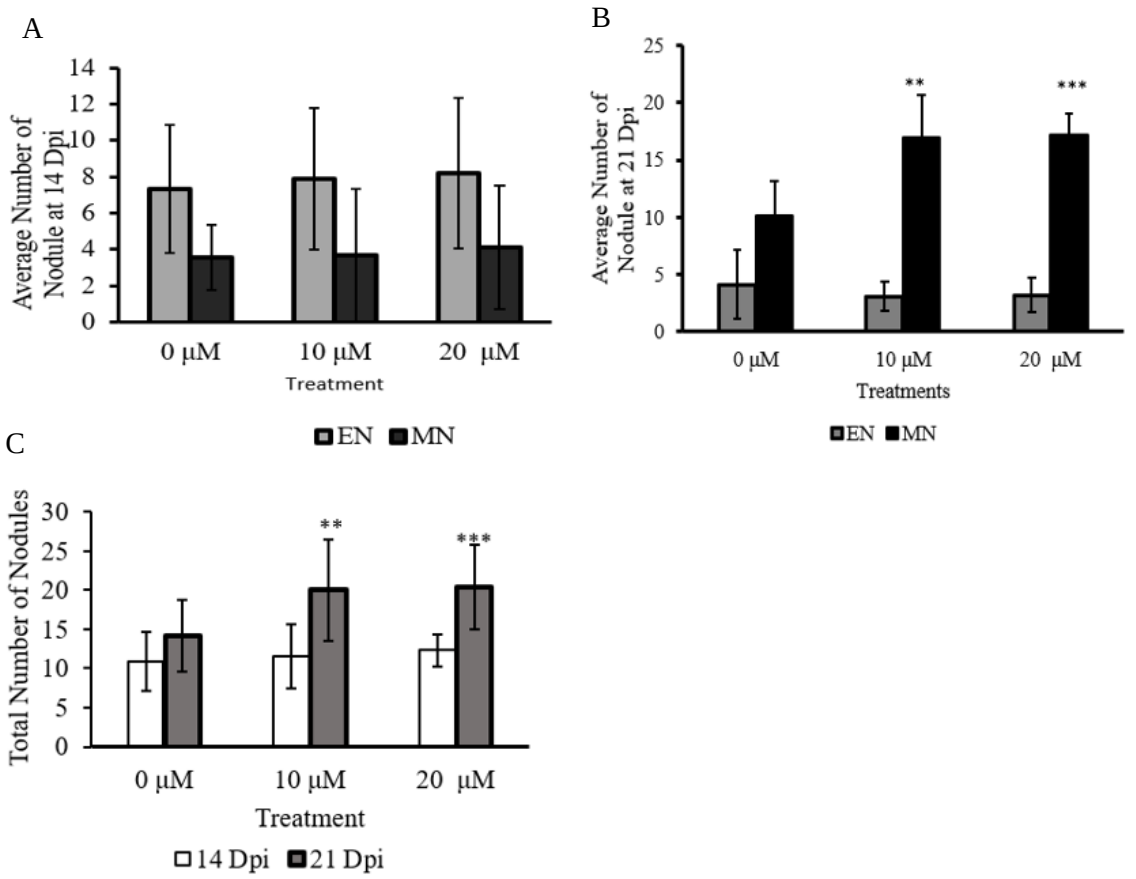

Figure 2.4-3: The effect of yucasin on soybean nodule development. (A) Numbers of emerging and mature nodules at 14 dpi. (B) Numbers of emerging and mature nodules at 21 dpi. (C) Total number of nodules in wild-type soybean plants treated with yucasin at 0µM, 10Mm, and 20µM concentration at 14 dpi and 21 dpi. Data are shown average of count from 27 plants in three biological replicates; Error bars indicate SD, Student's t-test. **-P<0.01 and *** -P<0.001.

To evaluate nodule marker gene expression RT-qPCR was performed on known nodule zone-specific marker genes, *ENOD2*, *ENOD40*, *NIN*, *FWL1*, and *NSP1*. *ENOD2* is known to be expressed in the nodule parenchyma region (VAN DE WIEL *et al.* 1990), *ENOD40* is known to be expressed in developing nodule primordium and in the uninfected cells of the infection zone in a matured nodule (YANG *et al.* 1993). *NIN* is a key transcription factor in nodule development and is essential for infection thread formation and generation of

nodule primordia in the cortex (SOYANO *et al.* 2015). *FWL1* is specifically expressed in root hair cells in response to rhizobia and infected cells of the nodules (QIAO *et al.* 2017). *NSP1* is crucial for nodule development and is responsible for the nodulation signaling pathway in legumes (SMITA *et al.* 2020). The expression of these marker genes in inoculated with USDA 110 and treated with different concentrations of yucasin soybean roots showed various expression levels (Figure 2.4-4). The expression levels of *FWL1*, *ENOD2*, *ENOD40*, and *NSP1* showed an increasing trend with increasing concentrations of yucasin, but the observed difference was not statistically significant However, the expression pattern of these marker genes at 10μM yucasin decreased when compared to control plants and this decrease was significant in the *ENOD2* marker gene. Thus, 20 μM yucasin treatment might consistently increase soybean nodulation.

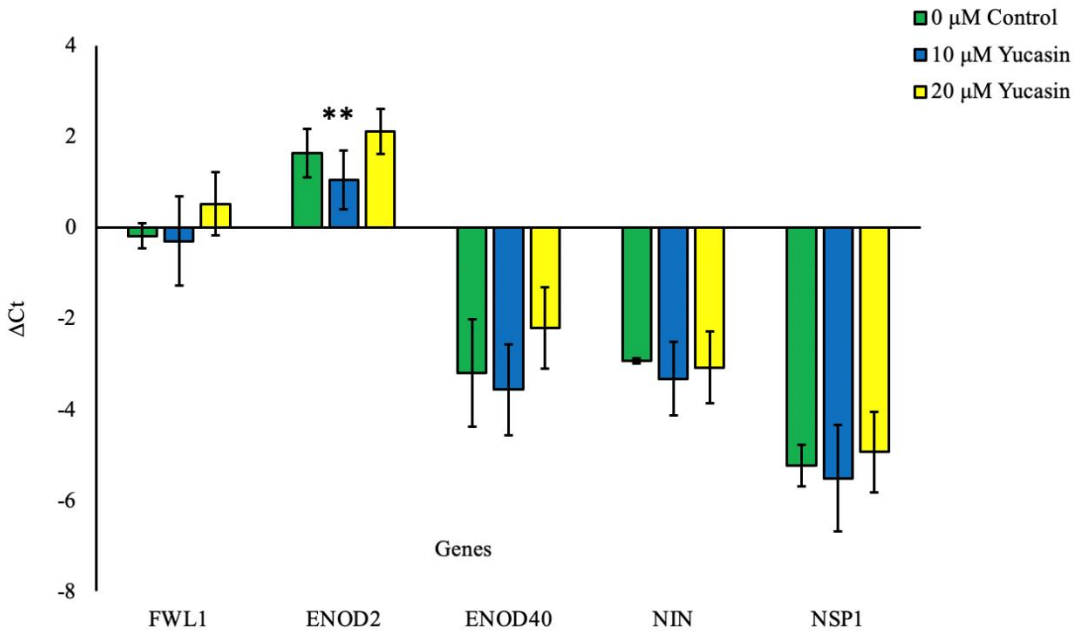

Figure 2.4-4: Expression patterns of marker genes FWL1, ENOD2, ENOD40, NIN, and NSPI at 21 dpi time point. The figure shows the difference in the expression pattern of nodulation marker genes by RT-qPCR. Delta Ct values (normalized to Actin) are plotted on the y-axis and marker genes at the x-axis. Data shows the average of 3 biological replicates; Error bars indicate SD, Student's t-test. **-P<0.01.

2.4.3 EFFECT OF YUCASIN ON NITROGENASE ACTIVITY

The nitrogenase enzyme is capable of reducing acetylene (C_2H_2) to ethylene (C_2H_4) and therefore, C_2H_2 can be used as an alternative substrate to measure nitrogenase activity. Plants were treated with 0 µM and 20 µM yucasin and grown in the growth chamber and greenhouse to identify nitrogenase activity in soybean nodules. Plants were harvested at 21 dpi and ARA was performed using gas chromatography for plants that were grown in the growth chamber. In the greenhouse, soybean plants treated with 0 µM and 20 µM yucasin were harvested for ARA after 6 weeks of inoculation. For both experiments, the

area of ethylene production was normalized per nodule of soybean roots and per plant. Nitrogenase activity per plant and nodule were unchanged by 20 µM of yucasin treatment for both experiments (growth chamber and green house) (Figure 2.4-5A and B and Figure 2.4-6 A and B).

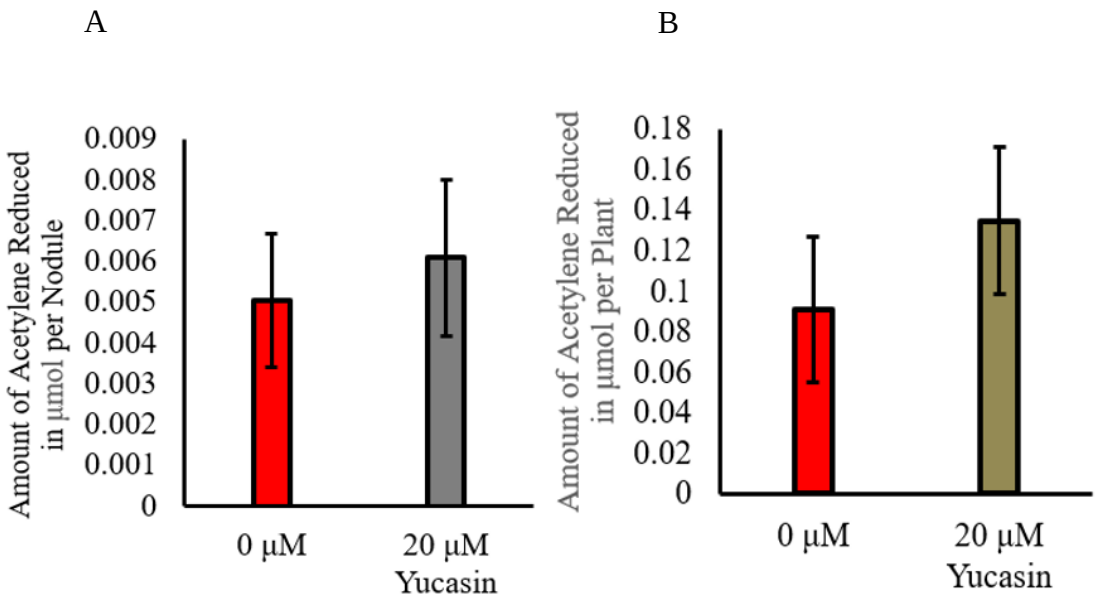

Figure 2.4-5:The effect of yucasin on Acetylene Reduction Activity of nodules formed in soybean roots, treated with yucasin at 0 µM and 20 µM and grown in the growth chamber. (A) Acetylene Reduction Activity per nodule. (B) Acetylene Reduction Activity per plant. Data are means of three biological replicates; Error bars indicate SD, Wilcoxon-Mann-Whitney test.

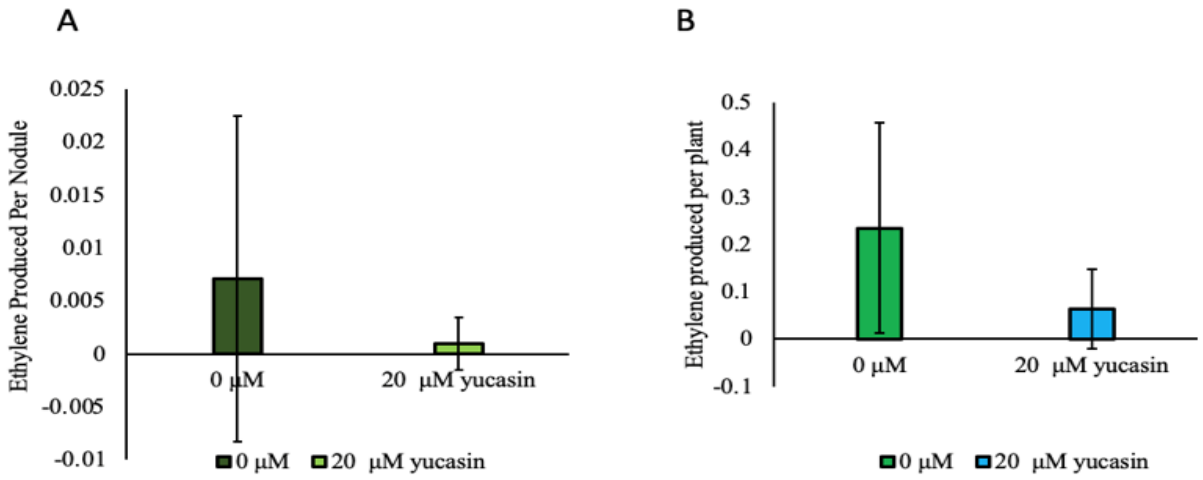

Figure 2.4-6: The effect of yucasin on Acetylene Reduction Activity of nodules formed in soybean roots, treated with yucasin at 0 µM and 20 µM and grown in the green house**.** (A) Acetylene Reduction Activity per nodule and (B) Acetylene Reduction Activity per plant. Data are means of three biological replicates; Error bars indicate SD, Wilcoxon-Mann-Whitney test for both.

2.4.4 EFFECT OF YUCASIN ON CHLOROPHYLL CONTENT INDEX

Chlorophyll is one of the important components of photosynthesis which plants use sunlight energy to produce sugars from water and carbon dioxide. The specific leaf nitrogen (SLN) content positively affects photosynthesis, which is partly related to nitrogen (N) partitioning in photosynthetic enzymes, pigment content, and the size, number, and composition of chloroplasts, and when leaf N content increase photosynthesis increases linearly. Thus, in the greenhouse experiment, the chlorophyll content index from plants treated with 0 µM and 20 µM of yucasin was measured. Results showed that yucasin treatment did not change the chlorophyll content index of plants (Figure 2.4-7).

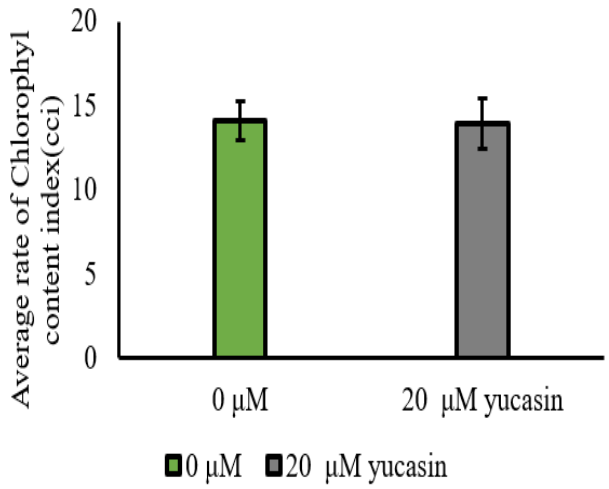

Figure 2.4-7: The effect of yucasin on soybean leaf chlorophyll content. Soybean plants were treated with yucasin at 0 µM and 20 µM and grown in the greenhouse. Data are means of three biological replicates; Error bars indicate SD, Student's t-test.

2.4.5 EFFECT OF YUCASIN ON GRAIN YIELD AND PROTEIN CONCENTRATION

To determine the effect of yucasin on grain yield, different grain yield components were measured. In soybean, grain yield components are defined by plant height (cm), the number of fertile nodes, pod number per plant, seed number per plant, seeds weight of plant(g), plant harvest index (%), and seed weight (g). In this experiment, pod number, seed number per plant, seeds weight of the plant, and seed weight per seed were measured as grain yield components which are important indicators of the grain yield level of soybean. Also, seed protein concentration was measured by Pierce Coomassie Plus (Bradford) Assay and all results showed that yucasin treatment did not significantly alter any of these traits (Figure 2.4-8,9 A and B, and Figure 2.4-10). Furthermore, yucasin did not affect the seed nitrogen content (Figure 2.4-11).

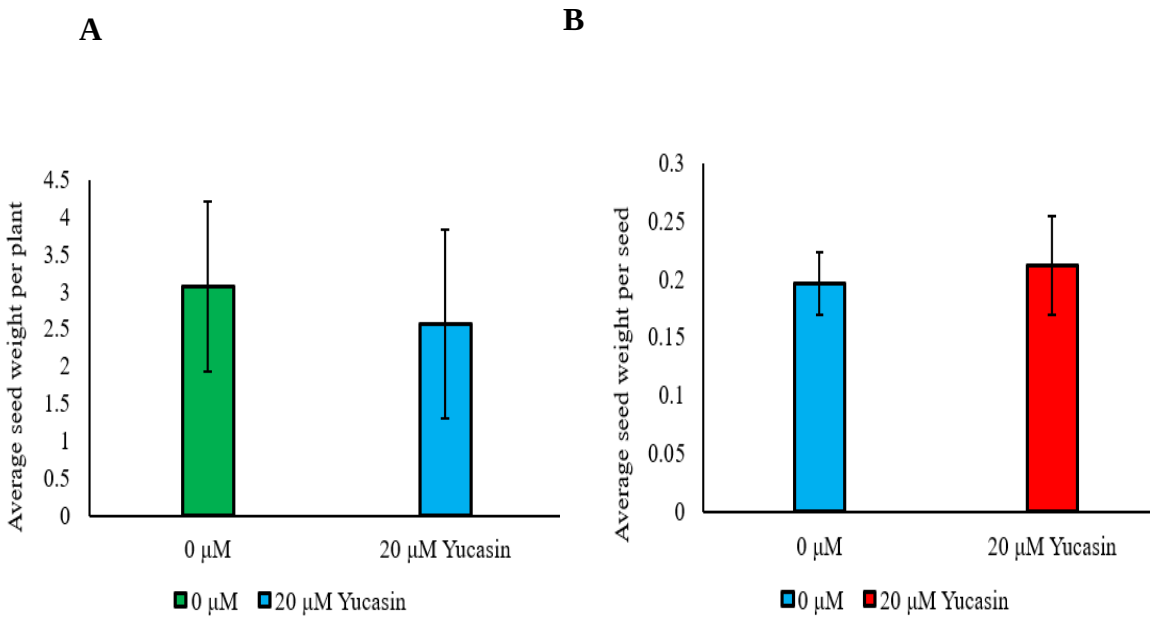

Figure 2.4-8: The effect of yucasin on soybean seed weight. Soybean plants were treated with yucasin at 0 µM and 20 µM and grown in the greenhouse. Data are means of three biological replicates; Error bars indicate SD, Student's t-test.

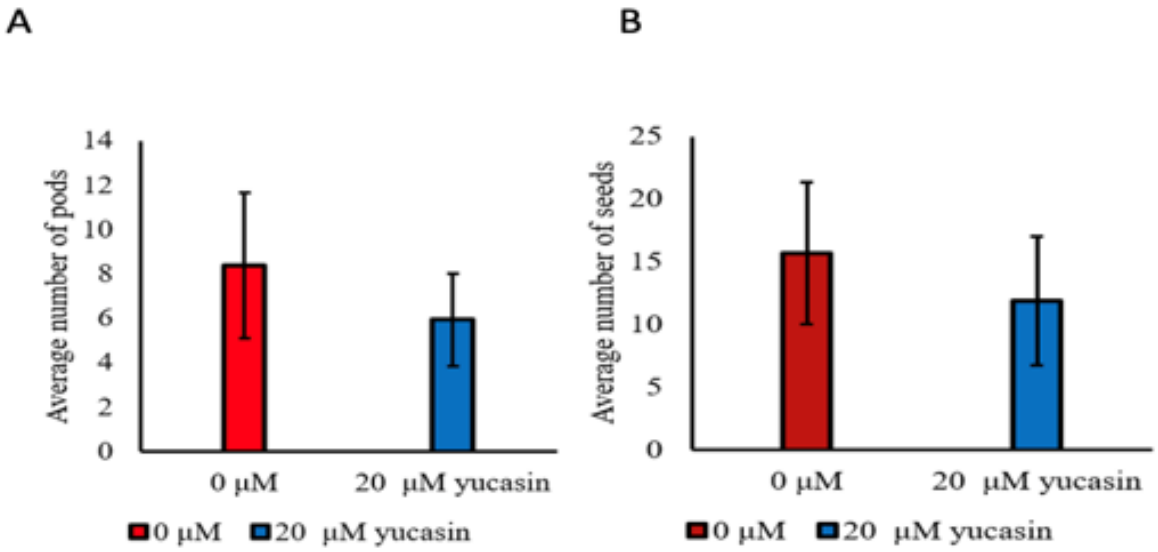

Figure 2.4-9:The effect of yucasin on the number of seed and pod measurements. Soybean plants were grown in the greenhouse were treated with yucasin at 0 µM and 20 µM. Data are means of three biological replicates; Error bars indicate SD, Student's t-test. Figure A shows the average number of pods per plant. Figure B shows the average number of seeds per plant.

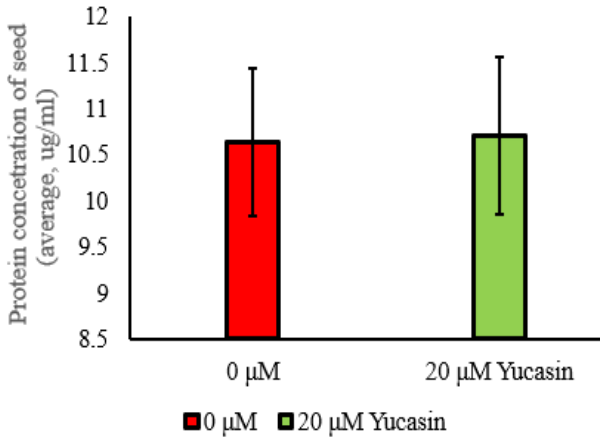

Figure 2.4-10: The effect of yucasin on soybean seed protein concentration. Soybean plants were treated with yucasin at 0 µM and 20 µM and grown in the greenhouse. Data are means of three biological replicates; Error bars indicate SD, Student's t-test.

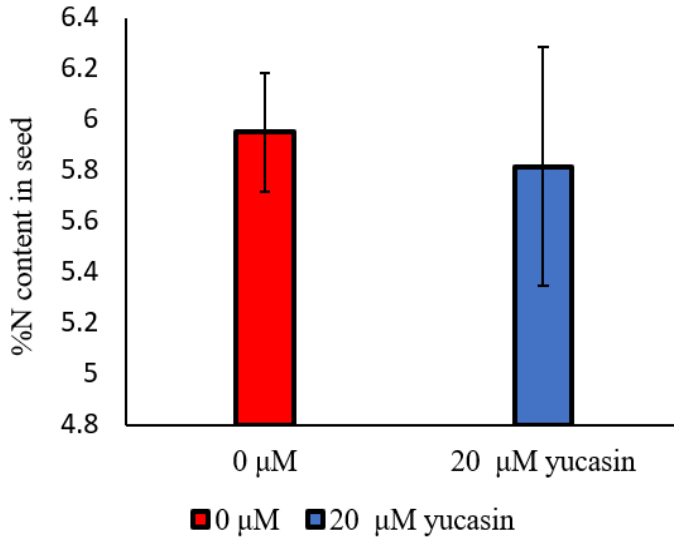

Figure 2.4-11: The effect of yucasin on soybean seed nitrogen concentration. Soybean plants were treated with yucasin at 0 µM and 20 µM and grown in the greenhouse. Data are means of three biological replicates; Error bars indicate SD, Student's t-test.

2.5 DISCUSSION

2.5.1 EFFECT OF YUCASIN ON SOYBEAN ROOT DEVELOPMENT

Auxin plays an essential role in plant growth and development (WOODWARD AND BARTEL 2005). In roots, the most well-known auxin-associated phenotypes are an increase in the number of lateral root primordia and a reduction in root length in a dose-dependent manner (OVERVOORDE *et al.* 2010). A low level of auxin concentration increases root length and a high level of auxin decreases root length (THIMANN 1939). It is clear that auxin plays a key role in lateral root formation (BHALERAO *et al.* 2002) but the inhibition of auxin signaling on lateral root formation is controversial as one study done by OVERVOORDE *et al.* 2010 showed that a higher level of IAA reduced strongly in the number of lateral roots (OVERVOORDE *et al.* 2010). Conversely, increased lateral root has been reported in plants

with high auxin content (REED *et al.* 1998). Our results also showed that inhibiting the auxin biosynthesis by yucasin application in soybean plants increased the root length of soybean plants and also increased the number of lateral roots as yucasin suppressed the activity of the YUCCA (YUC) gene family which are responsible for IAA synthesis in plants, resulting in the low level of auxin. According to WANG *et al.* 2019, changes in YUC gene expression levels have an important effect on auxin biosynthesis resulting in changes in root development. The increase in lateral root number can be due to increase in the root length of soybean plants under low levels of auxin. Therefore, we compared lateral root density calculated by dividing the number of lateral roots by the length to normalize for the effects of the treatment on length. In the case of our experiment, lateral root density was not significantly reduced by application of yucasin treatments.

Moreover, we wanted to see the effect of yucasin as an inhibitor of auxin biosynthesis on a different form of auxin such as IAA, IAA-ASP, IAA production affected by yucasin, and in soybean root. The effect of yucasin on the production of IAA was measured by NISHIMURA *et al.* 2014 and yucasin resulted in a decrease in the amount of IAA. We observed a reduction only in IAA-ASP. As root IAA pools are derived from a combination of local synthesis and shoot-to-root transport, it is possible that yucasin treatment only had a marginal impact limited to the root tip region.

We were able to evaluate gene expression of auxin-responsive markers *IAA1, IAA8, IAA9,* and *GH3* in response to yucasin treatment using qRT-PCR. A previous study performed in our lab found that the expression level of *IAA1* and *IAA9* in yucasin treated with soybean roots resulted in a significant reduction in the expression of these genes and these genes were downregulated in roots with suppressed auxin biosynthesis. In our study, the

expression level of *IAA1, IAA8, IAA9* showed a decreasing trend in roots treated with yucasin. This evidence indicated that yucasin treatment very likely reduced auxin levels in soybean roots.

2.5.2 EFFECT OF YUCASIN ON SOYBEAN NODULE DEVELOPMENT

Previous studies have emphasized the role of auxin in nodule organogenesis and rhizobia infection (BREAKSPEAR *et al.* 2014; WANG *et al.* 2019). Auxin transport may be the main contributor to increased auxin during nodule primordium during indeterminate nodule development, but there is little direct evidence for the involvement of auxin biosynthesis (WANG *et al.* 2019). We evaluated yucasin's role in regulating auxin levels and nodule development in soybean plants. The inhibiting of auxin biosynthesis in soybean root with yucasin increased the total number of the nodule and also the number of mature nodules at 21 dpi. Conversely, overproduction of IAA causes less nodule number and lower nodule mass in soybean plants in comparison to plants with wild-type rhizobia (HUNTER 1987; PII *et al.* 2007). We also observed that the suppression of auxin biosynthesis increased the expression levels of different nodulation marker genes, *FWL1*, *ENOD2*, *ENOD40* at 20 μM yucasin. This suggests that a low level of auxin in soybean root increases the nodule development affecting the expression of these genes.

2.6 CONCLUSION

Yucasin was used to investigate the potential role of auxin biosynthesis in soybean root and nodule development. Soybean root length was increased, IAA-ASP levels were reduced, and the expression of the different auxin marker gene, *IAA9*, was decreased significantly with the application of yucasin. These observations were consistent with the

reduction of IAA levels in the roots although the spatial patterns of reduction in IAA levels were not determined in this study. Additionally, the number of nodules was enhanced with the application of yucasin, and the expression of *FWL1*, *ENOD2*, *ENOD40* were increased. Yucasin treatment increased mature and total nodule number at 21 dpi. Therefore, auxin biosynthesis appears to affect both nodule formation and nodule maturation. However, yucasin treatment did not affect nitrogenase activity, seed nitrogen content, protein concentration, and grain yield. Our results suggest a key role for local auxin biosynthesis in root nodule formation and maturation in soybean. In addition, yucasin treatment provides an effective approach to increase the number of mature nodules in soybean.

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CHAPTER 3

3.THE ROLE OF THE AUXIN SIGNALING IN SOYBEAN ROOT AND NODULE DEVELOPMENT

3.1 ABSTRACT

Auxin is one of the most important phytohormones and regulates nearly all stages of plant development including the development of nodules during the symbiotic interactions between leguminous plants and nitrogen-fixing rhizobia bacteria. Auxin signaling mediates the regulation of gene expression at the site of auxin action. Auxin action is mediated by a group of transcriptional factors namely auxin response factors (ARFs) which bind conserved DNA elements named auxin response elements (AuxREs) and regulate gene expression. Auxin initiates an interaction between TIR1/AFB and Aux/IAA proteins, causing degradation of the Aux/IAAs and the release of ARF repression. In this research, we aimed to evaluate the role of auxin signaling in soybean root, nodule development, and nitrogen fixation using different concentrations of PEO-IAA (auxin signaling inhibitor). We hypothesized that reduction in auxin signaling would lead to more nodules and increased nitrogen fixation. We evaluated different marker genes using qRT-PCR assays to determine if PEO-IAA affected auxin sensitivity and nodule development. The root length of soybean plants treated with 100 μM PEO-IAA was significantly higher than in mock-treated soybean plants as well as other concentrations of PEO-IAA. Plants treated with 10 μM, 20 μM of PEO-IAA showed a significant increase in the total number of nodules at 21 dpi, but no change at 14 dpi. Consistently, seedlings treated with 10μM and

20μM PEO-IAA had an increasing trend of increased expression of *FWL1*, *ENOD2*, and *NIN*. Furthermore, the application of PEO-IAA did not affect the amount of nitrogen fixed per plant and per nodule in soybean and did not affect grain yield. Overall, 10μM and 20μM PEO-IAA treatment might consistently increase nodulation in soybean roots and this could help devise strategies to increase nodule number to enhance nitrogen fixation.

3.2 INTRODUCTION

Auxin plays a crucial role in the growth and many developmental processes in plants. It also has a key role in nodule development as auxin and cytokinin hormone in soybean nodule development play a naturally antagonistic role and the miRNA160 helps to regulate the auxin signaling process. Thus, they affect soybean root structure as well as the nodule formation process (TURNER *et al.* 2013). Auxin signaling mediates the regulation of gene expression at the auction site of auxin. There are three key components of the auxin signaling: the F-box TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB) auxin co-receptors, the Auxin/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressors, and the AUXIN RESPONSE FACTOR (ARF) transcription factors (WANG AND ESTELLE 2014; LAVY AND ESTELLE 2016). Auxin initiates an interaction between TIR1/AFB and Aux/IAA proteins, causing degradation of the Aux/IAAs and the release of ARF repression. The difference in the auxin responses results in auxin signaling protein diversity. There are 29 Aux/IAA proteins, 23 ARFs, and 6 members of the TIR1/AFB group found in Arabidopsis. Aux/IAAs are early auxin response genes that encode short-lived proteins, localized at the nucleus, with four conserved domains, namely I, II, III, and IV (TIWARI *et al.* 2001). The rate of Auxin degradation is increased along with the increase of auxin concentration (TIWARI *et al.* 2001). Domain I

plays role in the repression of Aux/IAA with the help of conserved ethylene response factor (ERF)– associated amphiphilic repression motif and recruits corepressors such as TOPLESS (BREAKSPEAR *et al.* 2014) and this domain is localized in the N-terminal region. The second domain of Aux/IAA (Domain II) is responsible for the instability of Aux/IAA proteins is a degron domain interacting with an F-box protein, TIR 1, component of SCF TIR1 ubiquitin ligase complex (LISCUM AND REED 2002). Domain III and IV known as PB1 domain of Aux/IAA which has a similar amino acid sequence with motifs III and IV of ARFs functioning in dimerizing with ARFs (TIWARI *et al.* 2004).

The SCFs are a subgroup of an E3 ubiquitin ligase and SCF complexes consist of an F-box protein providing substrate recognition, and ARABIDOPSIS SKP1 HOMOLOG1 (ASK1), S-phase kinase-associated protein 1 (SKP1), the scaffold protein CULLIN1 (CUL1), and the RING-BOX1 (RBX1) protein which promotes transfer of ubiquitin molecules to the substrate (LAVY AND ESTELLE 2016). $SCF^{TIR/AFB}$ complex binds the Aux/IAA substrate in the presence of auxin through the TIR1 or AFB F-box protein, referred to as auxin co-receptors (VILLALOBOS *et al.* 2012). TIR1/AFB proteins consist of an F-box motif and a leucine-rich repeat (LRR) domain.

ARFs (Auxin Response Factor) is a set of transcriptional factors that regulate the expression of auxin response genes and it mostly binds to auxin response elements (AuxRE) in promoters of these genes (GUILFOYLE AND HAGEN 2007). ARFs like many transcriptional factors consist of three different modular domains, namely an amino-terminal DNA-binding domain (DBD), a middle region that functions as an activation domain (AD) or repression domain (RD), and a carboxy-terminal dimerization domain (CTD)(GUILFOYLE AND HAGEN 2007). DNA-binding domain of ARFs contains two

subdomains, one is the Dimerization domain (DD) that is responsible for either homo- or heterodimerization with different ARFs families stabilizing ARFs structure during transcription and another is the B3 domain that assists ARFs to bind with DNA segments (AuxREs) in promoter with the flexibility (BOER *et al.* 2014). Moreover, ARFs can homodimerize with each other through DD to produce cooperative DNA binding and Dimerized ARFs are more stable than in monomer forms of ARFs. However, the monomer and dimer form of ARFs also relies on the orientation of AuxREs. Moreover, AuxREs may appear as simple elements either in the form of direct or palindromic repeats. However, ARFs form monomer indirect repeats; but they require to form dimers on palindromic AuxREs to bind stably.

ARFs and Aux/IAA proteins contain similar Phox and Bem1 (PB1) domains, referred to as protein-protein interaction domains. The PB1 domain of ARFs facilitates the formation of homodimerization of ARF-ARF and heterodimerization of ARF-Aux/IAA (GUILFOYLE AND HAGEN 2007). The ARF-Aux/IAA interaction with the PB1 domain causes the repression of ARF suppressing the transcription of auxin-responsive genes (TIWARI *et al.* 2003). In low auxin concentration in a cell, Aux/IAA proteins dimerize to and repress the ARFs dependent auxin signaling pathway by forming the dimer with the ARFs. Aux/IAA bind with activator ARFs forming heterodimer through protein-protein interaction and represses transcription of the target gene by recruiting TPL(TOPLESS), known as a co-repressor. However, in presence of high auxin concentration, the SCF family helps to degrade the AUX/IAA proteins (TIWARI *et al.* 2003; TIWARI *et al.* 2004). Auxin is perceived in this way in the cells and further signaling occurs with the participation of different proteins.

There are two types of auxin signaling pathways (i) the nuclear auxin receptors TIR1/AFB, which mediate both transcriptional and non-transcriptional responses, and (ii) the AUXIN BINDING PROTEIN1 (ABP1) pathway with an unclear function (HAN *et al.* 2020). The repressors of ARFs are capable of repressing auxin gene expression. To evaluate auxin signaling pathways in soybean nodule development, the application of PEO-IAA which is a novel auxin antagonist that binds to transport inhibitor response 1/auxin signaling F-box proteins (TIR1/AFBs) will suppress the expression of auxin-responsive genes. In the auxin signaling pathway, PEO-IAA, auxin signaling antagonist, bind TIR1/AFBS receptors and block their functions. Thus, we aimed in this study to test the effect of different concentrations of PEO-IAA in soybean root and nodule development.

3.3 MATERIAL AND METHODS

3.3.1 PLANT MATERIAL

Plant material is the same in 2.3.1 part.

3.3.2 PEO-IAA TREATMENT

To determine the effect of auxin signaling in soybean nodulation, PEO-IAA chemical was employed, and treatments were applied in the growth chamber and greenhouse. In the growth chamber, the plants were treated with different concentrations of PEO-IAA 0, 10, 20, and 100 μM prepared as described in (Nishimura et al. 2014). The plants were watered alternatively between PEO-IAA mixed with N- PNS and with N- PNS. Wild-type soybean plants were treated with the corresponding concentration of PEO-IAA at 3 and 7 days after sowing seeds. Plants were harvested at 10 days for root length, lateral roots count, lateral root density, and gene expression assays. The normality of the data was determined using the Shapiro Wilk test and the statistical significance was determined using t-test in R

version 3.3.0. As for studying the effect of nodulation with PEO-IAA treatment, the plants were inoculated with *B. japonicum* USDA 110 at 3 days after sowing seeds, the next day (1dpi) they were treated with the corresponding PEO-IAA solution and again at 5, 9, and 13 dpi. Plants were harvested at 14 and 21 dpi days to measure nodulation assay, gene expression, and ARA. In the greenhouse, plants were inoculated with *B. japonicum* at 7 days after sowing seed and two days later of inoculation, plants were treated with 0 and 20 μM PEO-IAA as selected concentration based on growth chamber experiment results. The treatments were continued once a week for 6 weeks. Plants were harvested at 6 weeks after inoculation to measure nitrogen fixation rate (ARA).

3.3.3 NODULATION ASSAY

The same method was followed in 2.3.4.

3.3.4 ACETYLENE REDUCTION ASSAY

The same method was followed in 2.3.5.

3.3.5 RNA EXTRACTION

The same method was followed in 2.3.6.

3.3.6 DNASE TREATMENT

The same method was followed in 2.3.7.

3.3.7 CDNA SYNTHESIS

The same method was followed in 2.3.8.

3.3.8 REVERSE TRANSCRIPTION-QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR)

The same method was followed in 2.3.9.

3.3.9 PROTEIN CONCENTRATION MEASUREMENT

The same method was followed in 2.3.10.

3.3.10 SEED NITROGEN CONTENT MEASUREMENT

The same method was followed in 2.3.11.

3.4 RESULTS

3.4.1 EFFECT OF PEO-IAA ON SOYBEAN ROOT DEVELOPMENT

To evaluate the effect of auxin signaling in soybean root development, PEO-IAA was used as a specific inhibitor of the $SCF^{TIR/AFB}$ -dependent auxin signaling pathway. Based on the previous studies, three different concentrations of PEO-IAA (10 μM, 20 μM, and 100 μM) were used to treat wild type soybean plants along with mock solvent control (0μM) as mentioned in 3.3.2. The seedlings were harvested at 10 days to analyze their root length, lateral roots, and lateral root density to measure the effect of potential reduction of endogenous IAA. The root length of soybean plants treated with 100 μM was significantly higher than in mock-treated soybean plants as well as other concentrations of PEO-IAA (Figure 3.4-1). Moreover, lateral roots count was significantly lower in 10 μM and 20 μM but there was no significant change in 100 μM of PEO-IAA. Lateral root density in plants treated with 100 μM is decreased significantly. (Figure 3.4-1). Based on our findings, PEO-IAA reduced likely auxin signaling in soybean.

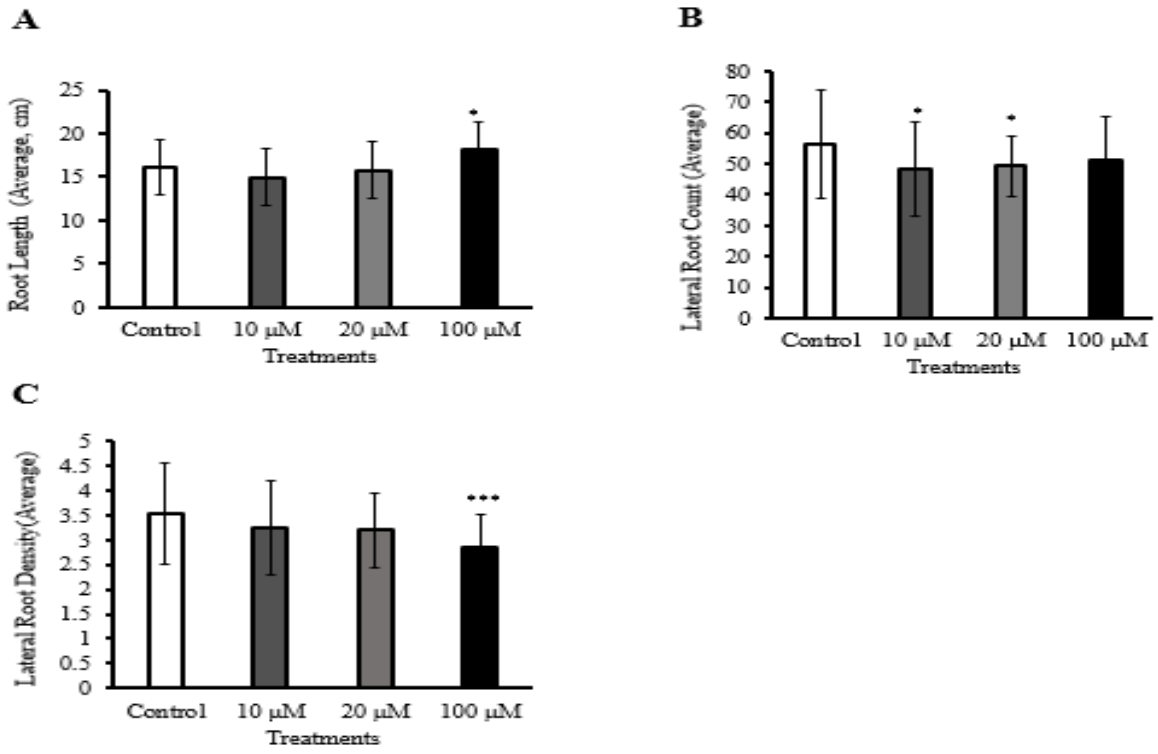

Figure 3.4-1 Effect of PEO-IAA on soybean root development (A, B &C). Root length (A) Lateral root count (B) and lateral root density (C; the number of lateral roots/ cm of root length) of wild type soybean plants treated with PEO-IAA at 0µM, 10µM, 20µM, and 100µM concentration for 10 days. Data are shown in A, B, and C are an average of 27 plants from three biological replicates; Error bars indicate SD, Student's t-test. * - P<0.05; *** -P<0.001.

Moreover, the effect of the auxin signaling inhibitor, PEO-IAA on the expression of auxin-responsive marker genes *IAA1*, *IAA8*, *IAA9*, and *GH3* was examined in roots using qRT-PCR. When the auxin output marker expression was analyzed PEO-IAA treated soybean roots, seedlings treated with different concentrations of PEO-IAA showed variable expression levels and it was not significant for all genes and concentrations. The concentration 10µM and 20µM of PEO-IAA had a significant increase of marker gene *IAA1*. Indeed, 10µM of PEO-IAA showed a consistent trend of increased marker gene expression for all auxin-responsive marker genes *IAA1*, *IAA8*, *IAA9*, and *GH3*(Figure 3.4-

2). The expression level of genes with 20µM of PEO-IAA showed varied responses (Figure 3.4-2). The effective concentration of PEO-IAA for auxin signaling differs depending on physiological measurement and gene expression study and it is still needed to investigate.

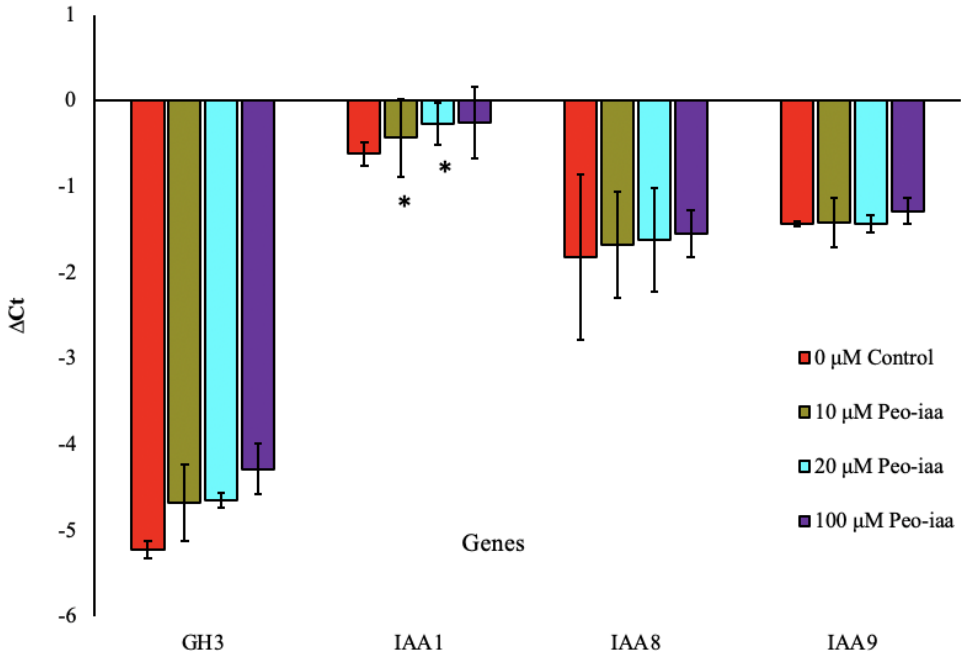

Figure 3.4-2: Expression patterns of marker genes GH3, IAA1, IAA8, and IAA9 at 10 days. Expression levels of auxin response marker genes, in wild type soybean plant roots treated with PEO-IAA at 0µM, 10µM, 20µM, and 100µM concentration for 10 days. Delta Ct values (normalized to Actin) are plotted on the y-axis and genes at the x-axis. Data shown are the average of 3 biological replicates; Error bars indicate SD, Student's t-test.
* -P<0.05.

3.4.2 EFFECT OF PEO-IAA ON SOYBEAN NODULE DEVELOPMENT

To determine the effect of auxin signaling on soybean nodulation, PEO-IAA compound was used in three different concentrations, 10 µM, 20 µM, and 100 µM (and plants were harvested at two different time points, 14 dpi, and 21 dpi, to measure the total number of

nodules, emerging nodules, and mature nodules. At 14 dpi, there was no significant change in emerging and mature nodule numbers in plants treated with PEO-IAA (Figure 3.4-3 A). However, plants treated with 10 μM, 20 μM of PEO-IAA displayed a significant increase in the number of mature nodules at 21 dpi (Figure 3.4-3 B). In a total number of nodules, plants treated with 10 μM, 20 μM of PEO-IAA showed a significant increase compared to those with 100 μM and mock-treated at 21 dpi, but no change at 14 dpi (Figure 3.4-3 C). There was no significant difference in the number of emerging and mature nodules at the high concentration of PEO-IAA tested, 100 μM. The effective concentration of PEO-IAA for various auxin responses may differ depending on the tissues. These all results indicated that different concentrations of PEO-IAA can work on different tissue and the suppression of auxin signaling by PEO-IAA led to increased nodulation in soybean plants.

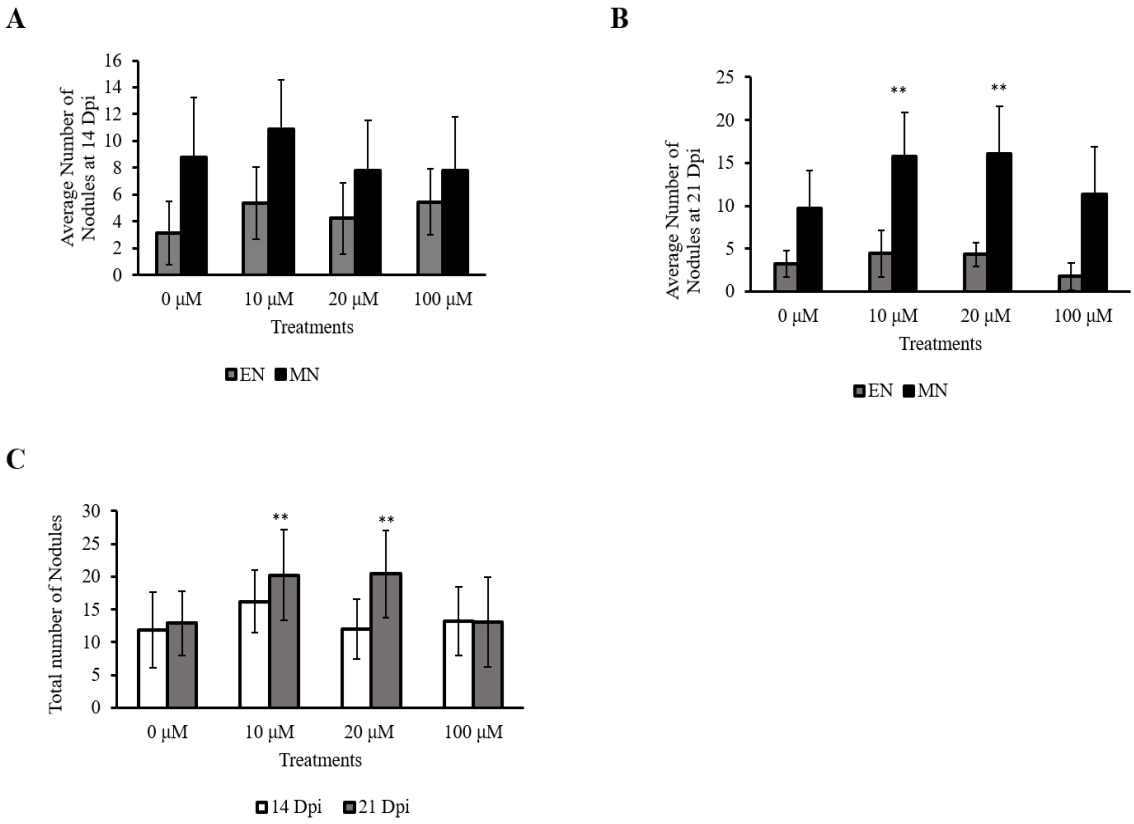

Figure 3.4-3: The effect of PEO-IAA on soybean nodule development (A, B & C). (A) Numbers of emerging and mature nodules at 14 dpi. (B) Numbers of emerging and mature nodules at 21 dpi. Data shown are an average of 27 plants from three replications; Error bars indicate SD, Student's t-test. **-P<0.01. C) Total number of nodules in wild type soybean plants treated with PEO-IAA at 0µM, 10µM, 20µM and 100µM concentration at 14 dpi and 21 dpi. Data shown are average of count from 27 plants in three biological replicates; Error bars indicate SD, Student's t-test. **-P<0.01.

The effect of PEO-IAA on the gene expression of nodule zone-specific marker genes was evaluated by using *ENOD2, ENOD40, NIN, FWL1,* and *NSP1* with the help of qRT-PCR. *ENOD2* is known to be expressed in the nodule parenchyma region (VAN DE WIEL *et al.* 1990), *ENOD40* is known to be expressed in developing nodule primordium and in the uninfected cells of the infection zone in a matured nodule (YANG *et al.* 1993). *NIN* is a key transcription factor in nodule development and essential for infection thread formation and

generation of nodule primordia in the cortex (SOYANO *et al.* 2015). *FWL1* is specifically expressed in root hair cells in response to rhizobia in nodules (QIAO *et al.* 2017). *NSP1* is crucial for nodule development and is responsible for the nodulation signaling pathway in legumes (SMITA *et al.* 2020). When nodule marker genes expression was analyzed in PEO-IAA-inoculated with USDA110 in soybean roots, seedlings treated with 10μM and 20μM PEO-IAA had the consistent trend of increased marker gene expression; in particular, there was a significant increase in the expression of *FWL1*, *ENOD2*, and *NIN* in 10μM PEO-IAA treatment (Figure 3.4-4). Seedlings treated with 100μM PEO-IAA treatment showed a varied response (Figure 3.4-4). This suggested that 10μM and 20μM PEO-IAA treatment might consistently increase nodulation in soybean roots.

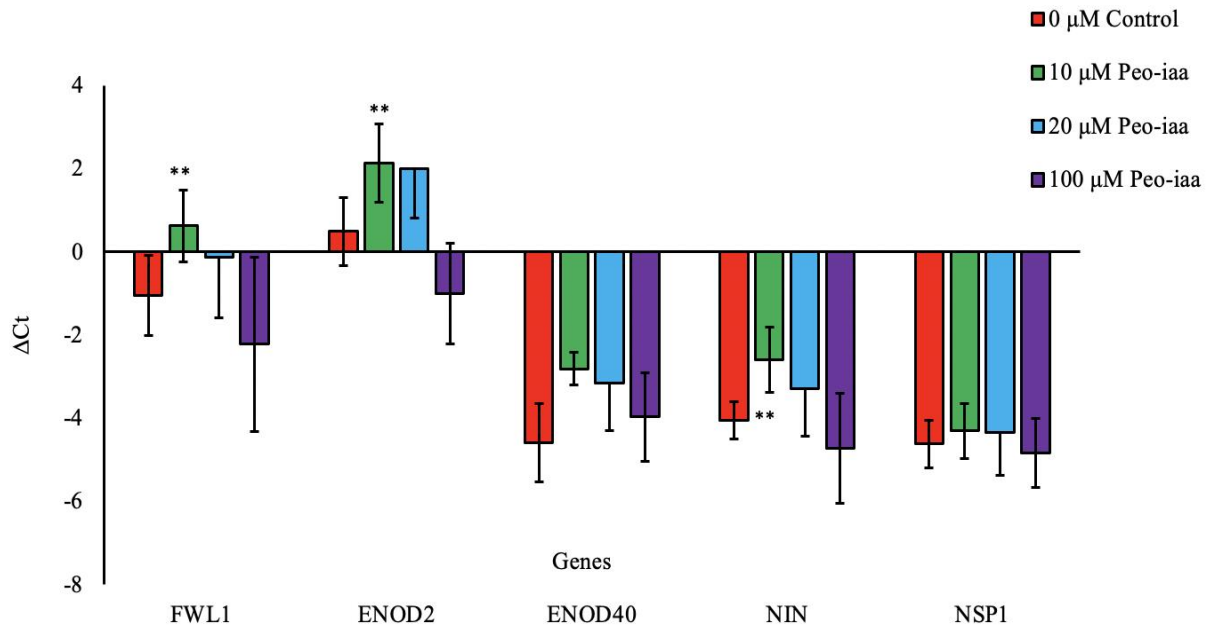

Figure 3.4-4: Expression patterns of marker genes FWL1, ENOD2, ENOD40, NIN, and NSPI at 21 dpi time point. The figure shows the difference in the expression pattern of nodulation marker genes by RT-qPCR. Delta Ct values (normalized to Actin) are plotted on the y-axis and marker genes at the x-axis. Data shows an average of 3 biological replicates; Error bars indicate SD, Student's t-test. **-P<0.01.

3.4.3 EFFECT OF PEO-IAA ON NITROGENASE ACTIVITY

Nitrogenase reduces acetylene (C_2H_2) to ethylene (C_2H_4) and can be used as an alternate assay to measure nitrogenase activity in soybean roots nodules treated with 0 µM and 20 µM of PEO-IAA in the growth chamber and greenhouse. Plants were harvested at 21 dpi and ARA was performed using gas chromatography for plants that were grown in the growth chamber. In the greenhouse, soybean plants treated with 0 µM and 20 µM PEO-IAA was harvested for ARA after 6 weeks of inoculation. The amount of ethylene production was normalized to per nodule of soybean roots and per plant. Plants treated with

PEO-IAA did not affect ethylene production (Figure 3.4-5, 6 A and B). Overall, these results can suggest that the application of PEO-IAA had no significant effect on nitrogenase activity.

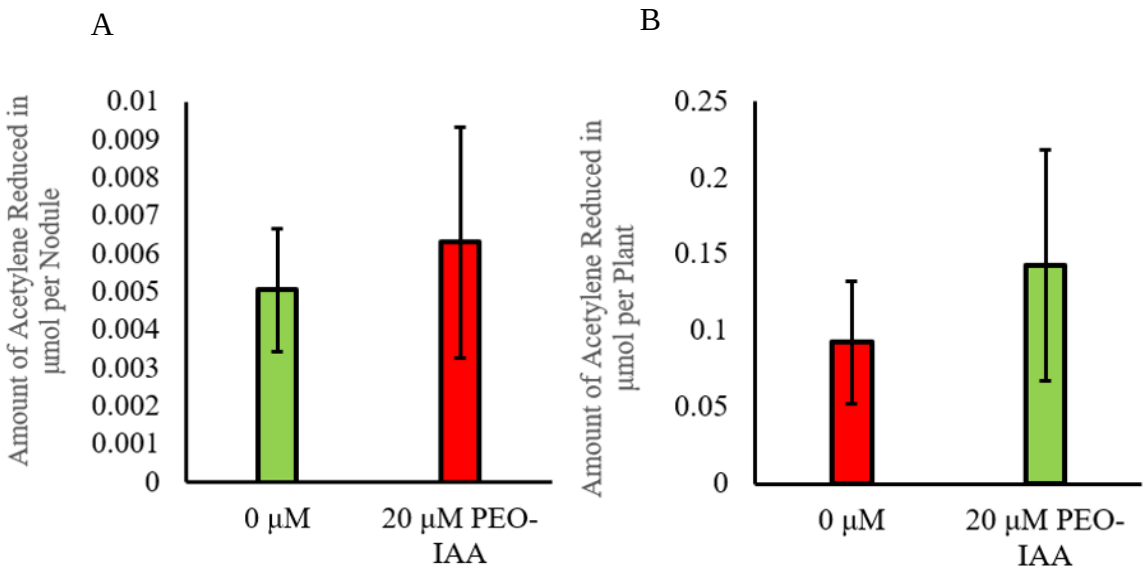

Figure 3.4-5: The effect of PEO-IAA on Acetylene Reduction Activity of nodules formed in soybean roots. Plants were treated with PEO-IAA (0 µM and 20 µM) and grown in the growth chamber. Data are means of three biological replicates; Error bars indicate SD, Wilcoxon-Mann-Whitney test. (A) Acetylene Reduction Activity per nodule. (B) Acetylene Reduction Activity per plant.

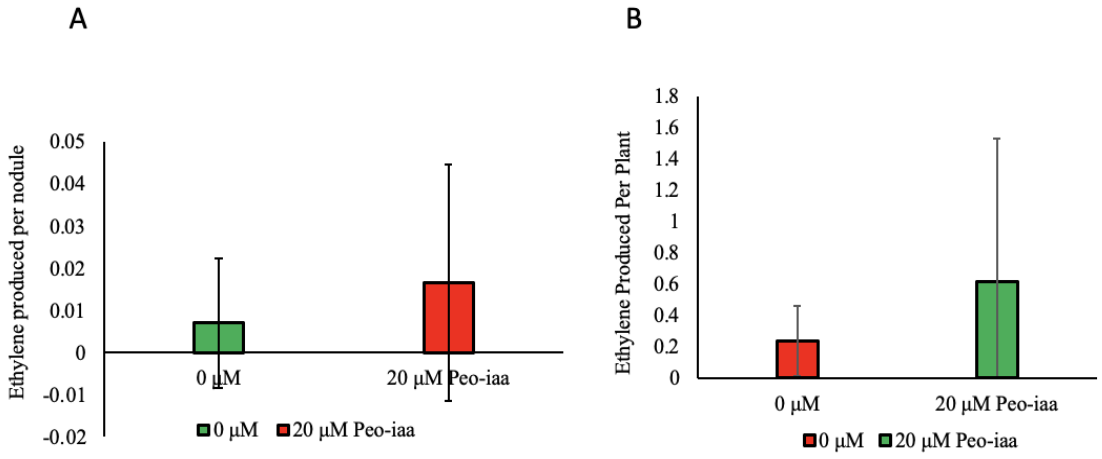

Figure 3.4-6: The effect of PEO-IAA on Acetylene Reduction Activity of nodules formed in soybean roots. Plants were treated with PEO-IAA (0 µM and 20 µM) and grown in the greenhouse. Data are means of three biological replicates; Error bars indicate SD, Wilcoxon-Mann-Whitney test. (A) Acetylene Reduction Activity per nodule. (B) Acetylene Reduction Activity per plant.

3.4.4 EFFECT OF PEO-IAA ON CHLOROPHYLL CONTENT INDEX

Photosynthesis is a crucial process of plant life, and it is influenced by different factors. Chlorophyll is one of the main components of photosynthesis. Nitrogen is a key component of chlorophyll; thus, the chlorophyll content index was measured in this study. Results showed that PEO-IAA treatment did not significantly affect the chlorophyll content of plants (Figure 3.4-7).

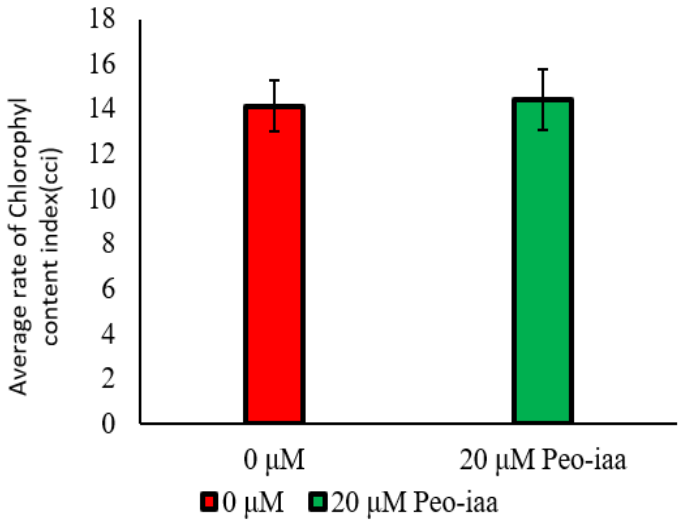

Figure 3.4-7: The effect of PEO-IAA on soybean leaf chlorophyll content. Soybean plants grown in the greenhouse were treated with PEO-IAA with 0 µM and 20 µM. Data are means of three biological replicates; Error bars indicate SD, Student's t-test.

3.4.5 EFFECT OF PEO-IAA ON GRAIN YIELD AND PROTEIN CONCENTRATION

To determine the effect of PEO-IAA on grain yield, different grain yield components such as pod number, seed number per plant, seeds weight of the plant, and seed weight per seed were measured. Also, seed protein concentrations were checked and all results showed that there was no significant difference between all traits treated with *0 µM and 20 µM* yucasin (Figure 3.4-8,9 A and B, and Figure 3.4-10). Moreover, yucasin did not affect the seed nitrogen content (Figure 3.4-11).

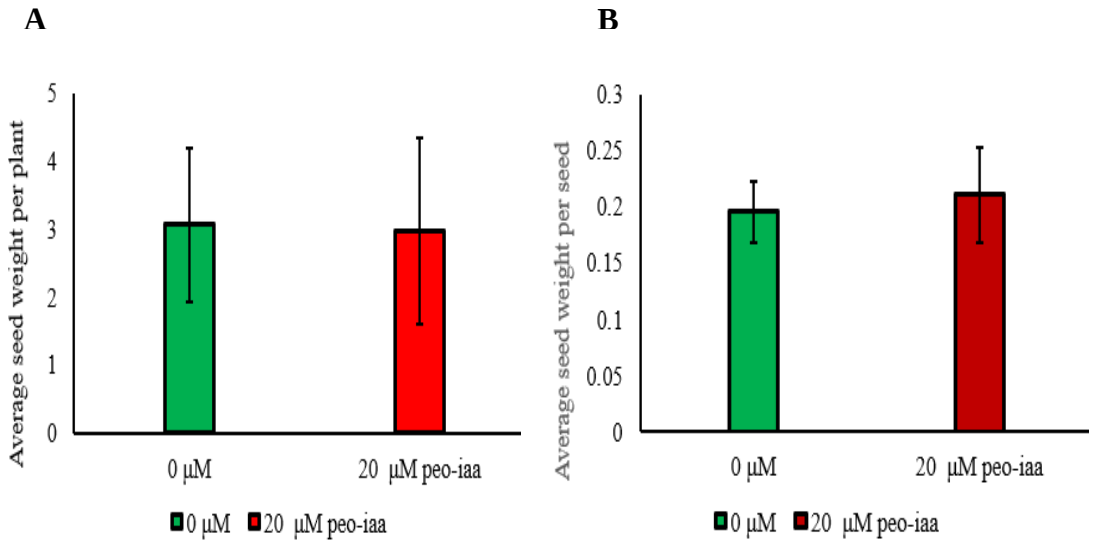

Figure 3.4-8: The effect of PEO-IAA on seed weight. Soybean plants grown in the greenhouse were treated with PEO-IAA at 0 µM and 20 µM. Data are means of three biological replicates; Error bars indicate SD, Student's t-test. Figure A shows an average seed weight per plant. Figure B shows an average weight per seed.

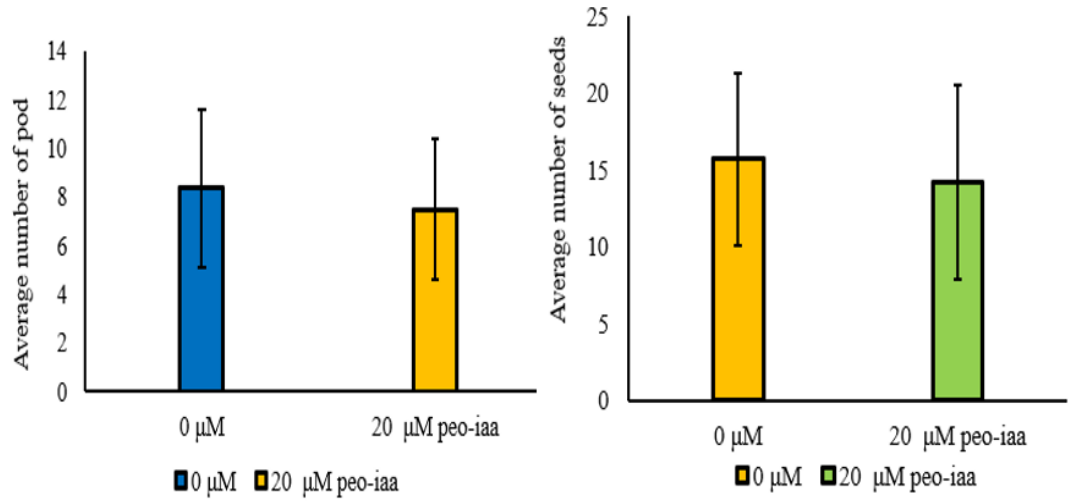

Figure 3.4-9: The effect of PEO-IAA on the number of seed and pod measurements. Soybean plants grown in the greenhouse were treated with PEO-IAA at 0 µM and 20 µM. Data are means of three biological replicates; Error bars indicate SD, Student's t-test. Figure A shows the average number of pods per plant. Figure B shows the average number of seeds per plant.

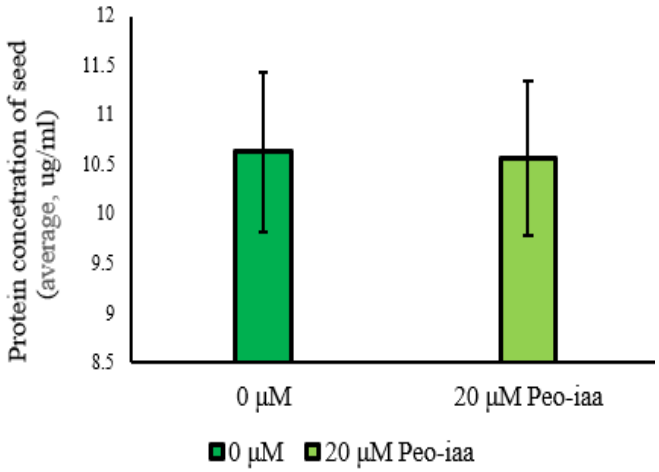

Figure 3.4-10: The effect of PEO-IAA on soybean seed protein concentration. Soybean plants were treated with PEO-IAA at 0 µM and 20 µM and grown in the greenhouse. Data are means of three biological replicates; Error bars indicate SD, Student's t-test.

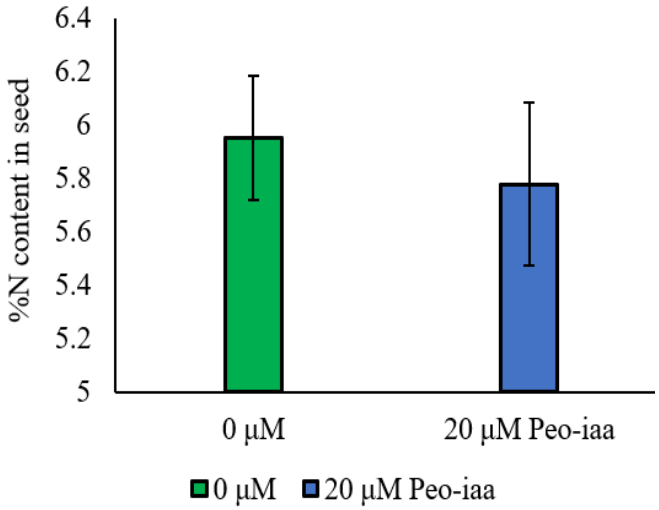

Figure 3.4-11: The effect of PEO-IAA on soybean seed nitrogen concentration. Soybean plants were treated with PEO-IAA at 0 µM and 20 µM and grown in the greenhouse. Data are means of three biological replicates; Error bars indicate SD, Student's t-test.

3.5 DISCUSSION

3.5.1 EFFECT OF AUXIN ANTAGONIST, PEO-IAA, ON ROOT DEVELOPMENT

PEO-IAA, a newly synthesized auxin antagonist, binds to TIR1/AFBs receptors and blocks their functions resulting in an auxin-defective phenotype. Our first observation was related to the growth of phenotype in the presence of different concentrations of PEO-IAA applied on soybean roots. At 100 μM, soybean root elongation was significantly enhanced compared to plants with treated 0μM, 10μM, and 20μM. However, according to TAKANASHI *et al.* 2011, root elongation in *L. japonicus* was slightly enhanced at 10 μM. The difference between our results can be due to the growing media and harvesting time as in our study we used potting mixture (vermiculite and perlite in the ratio of 1:3) and harvested the plant at 10 days but they grew *L. japonicus* in agar plates and measured root length at 5 days. Moreover, they found out that the higher concentration of PEO-IAA has inhibited the root growth on agar plates. We also found that at 100 μM of PEO-IAA concentration, lateral root density (LRD) was significantly decreased as compared to that of control concentration 0μM, 10μM, and 20μM. Moreover, we also investigated the gene expression pattern of auxin-responsive markers, *GH3, IAA1, IAA8,* and *IAA9* in roots using qRT-PCR to confirm the effect of PEO-IAA since PEO-IAA can block auxin functions. However, the results were highly different than expected as in our hypothesis we stated that PEO-IAA would reduce auxin signaling. In our result, the expression of auxin marker genes, *GH3, IAA1, IAA8,* and *IAA9,* was increased with the application of PEO-IAA. These results were not supporting measurements related to roots such as root length and LRD. In this study, we used three biological replications for gene expression study and the number

of replications might not be sufficient to detect the significance of gene expression level. Thus, increasing the biological replication number could enhance our results. This is an unexpected expression pattern and needs to be studied further to have a clear understanding of its role during soybean root development.

3.5.2 EFFECT OF AUXIN ANTAGONIST, PEO-IAA, ON NODULE DEVELOPMENT

The nodulation process in indeterminate and determinate nodules is well studied among several types of nodules in legume plants. Previous studies suggested auxin is involved in nodulation and it is well known especially in indeterminate nodules. However, the role of auxin in the development of determinate nodules is still unknown. Since PEO-IAA inhibits functions of auxin as α-alkyl-IAA competitively binds to the auxin-binding site of receptor molecules TIR1/AFBs. Therefore, in this study, we investigated the effect of PEO-IAA on nodulation in soybean roots. The number of nodules and number of the mature nodule was significantly higher at 10μM and 20μM at 21 dpi as compared to those with 0μM and 100μM. The effect of 10μM concentration of PEO-IAA on nodule development has been demonstrated in *L. japonicus* in vermiculate media (TAKANASHI *et al.* 2011). We also investigated the expression of different genes (*ENOD2*, *ENOD40*, *NIN*, *FWL1*, and *NSP1*) associated with soybean nodule development. The expression level of marker genes increased with the application of 10μM and 20μM PEO-IAA. Especially, 10μM PEO-IAA increased significantly the expression of marker genes, *FWL1*, *ENOD2*, and *NIN*. At 100μM PEO-IAA treatment showed different responses according to marker genes. For example, the marker genes *NIN* and *FWL1* showed lower expression levels compared to control plants. According to TAKANASHI *et al.* 2011, the effective concentration of PEO-

IAA can change depending on the plant tissues; for example, the inhibition of lenticel formation was seen at 100μM PEO-IAA. Here, the application of 100μM PEO-IAA decreased the expression level of *NIN* which is essential for infection thread formation and generation of nodule primordia in the cortex. This might suggest that the higher concentration of PEO-IAA can block the nodulation in soybean plants and distinct auxin involvement affect the determinate nodule development.

3.6 CONCLUSION

Nodule development is regulated by different phytohormones such as auxin. In this study, the role of auxin signaling during nodule development in soybean was evaluated using an auxin perception inhibitor, PEO-IAA. We found that blocking the auxin signaling affected soybean nodulation and altered the expression level of different nodulation genes and the number of nodules. Furthermore, PEO-IAA treatment increased nodule number especially mature nodule number at 21 dpi, however, it did not affect nitrogenase activity, seed nitrogen content, seed protein concentration, and grain yield. According to our results, auxin signaling plays an important role in soybean nodule maturation and root nodule formation. Thus, these results indicate that auxin is required for the normal of determinate nodules in a multidirectional manner.

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CHAPTER 4

4. 1 CONCLUSION

Auxin plays important role in most plant growth and development processes including legume nodule development. Although auxin plays crucial role in nodulation, the specific roles of auxin biosynthesis and auxin signaling need to be investigated. We evaluated the effect of yucasin (an auxin biosynthesis inhibitor) and PEO-IAA (auxin signaling inhibitor) on soybean nodule development.

Yucasin treatment (10 and 20 μM) significantly increased the root length and lateral root count, but not lateral root density in soybean. Consistent with this the expression of three auxin-response marker genes showed a decreasing trend of gene expression with increasing concentrations of yucasin treatment. However, the application of 20 μM yucasin treatment led to a reduction in the inactive storage form of auxin (IAA-Asp), but not the free form (IAA). Although 10 μM yucasin treatment led to the observed physiological and gene expression responses, we observed no measurable reduction in free or conjugated forms of IAA. Two potential explanations exist for this apparent discrepancy: (1) It is likely that yucasin treatment reduced auxin levels only in specific tissues of the root where YUCCA genes are most active e.g., the root tip (CAO *et al.* 2019) resulting in longer root length, but no overall reduction in IAA levels throughout the root; and (2) Root IAA pools are a combination of local biosynthesis and transport from the shoot, and therefore it is possible that the short-term yucasin treatments were not sufficient to significantly inhibit IAA biosynthesis in the shoot.

Auxin signaling inhibitor, PEO-IAA at 100 μM resulted in a significant increase in soybean root length and significantly reduced lateral root density consistent with the known physiological role for auxin signaling in these processes. Surprisingly, PEO-IAA treatment led to a higher expression and/or a trend of increasing expression of auxin-responsive marker genes. This needs to be investigated further. In summary, the results suggested that yucasin and PEO-IAA treatments led to the expected reduction in overall auxin physiological response in the roots. While the reduction in auxin levels by yucasin was supported by both molecular response and biochemical IAA measurement assays, a reduction in auxin response by PEO-IAA was not supported by molecular response assays.

Both yucasin and PEO-IAA treatments (10 and 20 μM) increased nodule number especially mature nodule number at 21 dpi. This indicated that suppression of auxin biosynthesis by yucasin or reduced auxin response by PEO-IAA treatment led to increased nodule formation. This observation was consistent with our previous report of increased nodule number in miR160-suppressed-soybean-roots with reduced auxin sensitivity. However, we observed a higher number of emerging nodules in miR160-suppressed soybean roots at 21 dpi while we observed a higher number of mature nodules at 21 dpi here. This apparent discrepancy might be due to the constant effects of genetic modification versus an oscillating effect of chemical treatments on auxin response/output. Despite the increase in the number of mature nodules, yucasin or PEO-IAA treatments did not affect nitrogenase activity, plant growth parameters, grain yield, or seed nitrogen/protein content. A potential explanation for no change in grain yield is that increasing mature nodule number or root length and having more root branches does not necessarily mean increased nitrogen nutrition in the shoot. Nitrogen demand in the shoot is what drives nutrient uptake and

nodule nitrogen fixation(CARTER AND TEGEDER 2016). Therefore, the increased nodule number provides an opportunity for increased nitrogen fixation if and when there is a demand from the shoot. Alternatively, it is possible that the increase in root growth and nodule number caused a source competition with the grain for carbon. These possibilities could be evaluated by performing these experiments in high-yielding soybean cultivars with maximal yield potential.

This study has identified chemical treatments with which mature nodule numbers can be manipulated as needed. This provides us a handle to optimize nodule number and syncing it with growth stages with high nitrogen demand or high yielding soybean genotypes that might demand high nitrogen fixation.

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5. APPENDICES

5.1 APPENDIX A: COMPOSITION OF HOAGLAND SOLUTION

Concentration for stock solution (1L)

	Component	**Mol.wt**	**Molarity (mM)**	**Amount (gm)**
Solution -I	$Ca(NO_3)_2.4H_2O$	236.15	892.7614	210.8253
Solution -II	$MgSO_4.7H_2O$	246.5	500	123.25
Solution-III	KNO_3	101.1032	1250	126.37
	KH_2PO_4	174.2	200	34.85
Soultion-IV	$Na_2FeEDTA$	372.24	11.5	4.28
Soultion-V	$MnCl_2$	125.84	3.6	0.453
	$ZnSO_4$	161.47	0.34	0.054
	H_3BO_3	61.83	11.5	0.711
	$CuSO_4$	159.6	0.125	0.0195
	H_2MoO_4	85%		0.085

Concentration for final solution (1L)

	Volume used (ml)	Final molarity(mM)
Solution -I	5.6	5
Solution -II	4	2
Solution-III	4	5
Soultion-IV	8	0.092
Soultion-V	4	1x

5.2 APPENDIX B: COMPOSITION OF NITROGEN FREE PLANT NUTRIENT SOLUTION

A. Macronutrient stocks:

Stock	**Stock vol**	**Amount(gm)**	**ml Stock/liter PNS**
$MgSO_4.7H_2O$	200ml	(12.3g)	2
$CaCl_2.2H_2O$	400ml	(29.4g)	4
$K_2HPO_4.3H_2O$	100ml	(3.4g)	1
K_2SO_4	400ml	(22.0g)	4
$FeCl_3.6H_2O$	250ml	(0.62g)	2.5

B. Micronutrients (10000x)

Stock	gm per 1 liter
H_3BO_3	1.42
$MnSO_4. H_2O$	0.77
$ZnSO_4.7H_2O$	1.73
$CuSO_4.5H_2O$	0.37
$NaMoO_4.2H_2O$	0.24
$CoCl_2.6H_2O$	0.025
$NiSO_4$	0.01