Effects of the Brown Seaweed, *Ascophyllum nodosum*, on the Nodulation and Growth of Alfalfa

by

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Abstract

The effect of *Ascophyllum nodosum* extracts on the nodulation and growth of alfalfa was investigated. Plant growth assay revealed that alfalfa treated with 2 g L^{-1} ANE exhibited a significant increase in leaf area. Under salt stress, alfalfa treated with 0.5 g L^{-1} ANE exhibited a significant increase in total length compared to controls. A root hair deformation assay indicated that ANE 0.5 g L^{-1} stimulated the synthesis of Nod factors secreted by rhizobia thus accelerate root hair deformation of alfalfa. Similarly, ANE 0.5 g L^{-1} caused an increase in *nodC* gene expression suggesting that ANE may act similarly to flavonoids in the *rhizobium*-legume symbiosis. Under field conditions, ANE increased the total number of functional nodules, total root length and total leaf area. Taken together, the results suggest that ANE may contain compound(s) that promote specific metabolic pathway both in alfalfa and bacterium thus enhance the symbiotic relationship.

List of Abbreviations and Symbols Used

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ANE	*Ascophyllum nodosum* extract
AVG	aminoethoxyvinylglycine
ANOVA	Analysis of variance
β	beta
BNF	biological nitrogen fixation
^{o}C	degree Celsius
CCRH	colonized curled root hairs
cm	centimeter
cm^2	square centimeter
EtOH	ethanol
g	gram
$g\ L^{-1}$	gram per liter
h	hour
ha	hectare
IAA	Indole-3-acetic acid
Kg	kilogram
L	liter
LANS	Long Ashton standard nutrient solution
LCOs	lipochitoologosaccharides
HSD	Honestly significant differences

µg	microgram
µl	microliter
µM	micromolar
µmol m^{-2} s^{-1}	micormoles per square meter per second
M	molar
m	meter
mg	milligram
min	Minute(s)
mL	milliliter
mm	millimeter
mM	millimolar
N	nitrogen
NFP	Nod factor perception
nm	nanometer
ONPG	ortho-nitrophenylgalactoside
OD	optical density
P	P value
rpm	revolutions per minute
SDS	Sodium dodecyl sulfate
Tg	teragram
w/v	weight/volume
%	percent
s	second(s)

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Chapter 1.0 Introduction

Nitrogen is an essential nutrient and a major component of amino acids, proteins, nucleic acids and nitrogenous metabolites. In nature, N exists in a number of different forms, such as nitrogen gas (N_2), ammonium (NH_4^+), nitrate (NO_3^-), and organic forms (Galloway 1998). Earth's atmosphere is composed of about 78% N_2. However, N_2 is not usable by plants and animals, but has to be transformed to readily available forms. Sufficient NH_4^+ and NO_3^-, when present in the soil, can improve the growth of plants and stimulate green biomass production of crops. The most common means of N addition to crops is through application of N fertilizers. However, only 20-50% of N applied in fertilizers is used by crop plants (Brady and Weil 2002), while the rest is lost in the form of volatilization, denitrification, runoff, and leaching, thus causing risk of environmental pollution.

In the absence of human activities, in the natural world, reactive N is created by biological nitrogen fixation (BNF) and lightning (Galloway 1998). BNF was first discovered by Beijerinck in 1901. It is a process in which atmospheric nitrogen is reduced to ammonia in the presence of the nitrogenase enzyme, by a specialized group of microorganisms, such as the symbiotic *Rhizobium* and *Sinorhizobium*, or the free-living soil bacteria, such as *Azospirillum* and *Azotobacter* (Wagner 2011). BNF provides about 90-130 Tg N year $^{-1}$ (Tg = 10^{12} g) in terrestrial ecosystems. This process is significantly altered by human activities and more N is mobilized by fertilizer production, legume and rice cultivation, and fossil fuel production (Galloway et al. 1995). The amount of N fixed by BNF depends on the extent of area planted to a specific crop and the fixation rate per hectare (Galloway 1998). The fixation rate is affected by soil conditions, such as

excessive soil moisture, drought, soil acidity, excess mineral N, and deficiency of necessary elements, by climate factors, such as temperature and light, and by biotic factors, such as the absence of the required rhizobia species, crop competition, and insects and nematodes damage (Al-Falih 2002; Liu et al. 2011).

BNF represents N gain and determines N fertilizer savings in the agriculture. The fixation rate is limited by factors mentioned above. However, there are common approaches to enhance BNF, such as inoculation with proven strains, host-plant screening and breeding, and adoption of cultural practices (Asadi et al. 2011; Eivazi et al. 2012).

Seaweed extract has been used as a natural organic fertilizer in agricultural system due to its effective and nutritious benefits to crops. Seaweed fertilizers can be used by plants in a short time and have little negative impact on humans, animals and the environment (Sathya et al. 2010). Previous studies have reported that the application of a brown seaweed extract, the extract of *Ascophyllum nodosum* (ANE), to legumes improves their growth, yield, nutrient uptake, and biochemical composition (Rathore et al. 2009; Fan et al. 2011). This improvement could be due to the stimulation of rhizosphere microbial diversity and activity by the application of seaweed extracts (Alam et al. 2010). The benefits of seaweed extract application could also be due to, at least in part that the carbohydrates, plant hormones, hormone-like plant growth regulators, trace elements, vitamins and amino acids contained in ANE (Khan et al. 2009). These results suggest that the ability of nitrogen fixation of legumes could be improved by the application of ANE.

Chapter 2.0 Literature Review

2.1 *Medicago sativa* (Alfalfa)

There are more than 60 species in the genus *Medicago* and one-third of these species are perennials (Silva et al. 2007). The most widely cultivated perennial species is alfalfa (*Medicago sativa*). Alfalfa was introduced to the Americas by Spanish colonizers in the 16th century (Galán Saúco and Cubero 2011). It resembles clover with clusters of small purple or purple-yellow flowers and coiled, nonspiny fruits; the plant can grow up to 1 m in height (Small and Jomphe 1989). Alfalfa is widely cultivated as a forage crop in both pure and mixed stands (www.medicago.org). Alfalfa has the ability to form associations with the nitrogen fixing rhizobium, *Sinorhizobium*, which can take nitrogen from the atmosphere and make it available to the plants.

In Nova Scotia, the numbers of sheep and goat have been increased in recent years. Nova Scotia has about 60% of the Atlantic herd for both sheep and goat (Statistics Canada 2006). Therefore alfalfa production has also increased, in order to meet the demand for the animal feed. Thus, alfalfa plays an important role in the agriculture.

2.2 Sources of Nitrogen

Nitrogen can be incorporated into an agro-ecosystem from different sources. Human activities mobilized about 140 Tg N year^{-1} by fertilizer production by the mid-1990s (Galloway 1998). Early in the twentieth century, German chemist Fritz Haber discovered a reaction that could transform atmospheric N_2 to NH_4^+. This process was purchased by a chemistry factory in Germany and Carl Bosch was assigned to make this reaction usable in the industry. The Haber-Bosch process synthesizes large amounts of N fertilizer,

boosting food production (Smil 2001; Crews and Peoples 2004). Over application of nitrogen fertilizer results in nitrogen build up in the soil. Atmospheric nitrogen can be deposited as N dioxide, nitric acid and particulates, or it can be dissolved in vapor and deposited through precipitation (Ledgard 2001). Wastes, for instance, manure, crop residues and compost, are other sources of nitrogen imported into soils (Power and Schepers 1989). There are also naturally occurring sources of nitrogen through biological N fixation, mobilization of N from soil organic matter by mineralization, and lightning. It is reported that bacteria in the soil, water and air on earth convert approximately 100 x 10^9 kg of atmospheric N annually into life sustaining compounds (Sprent and Sprent 1990).

2.3 Nitrogen Cycle

There are numerous forms of nitrogen in the environment. These include gaseous nitrogen (N_2), ammonium (NH_4^+), nitrate (NO_3^-), and organic nitrogen. While it is abundant on the surface of the earth, less than 2% is available to organisms (Galloway 1998). Although 78% of the earth's atmosphere is N_2 gas, it is a very stable molecule due to the triple bonds between the two N molecules. It must be transformed to available forms, like nitrate or ammonium, in order to be used by most of organisms. Nitrogen in the air or in the soil goes through a range of chemical and biological transformations, which can be combined into living and non-living materials, and released back to the atmosphere or the soil. This continuing cycle is referred to as the Nitrogen Cycle (Figure 2.1). The biochemical exchanges involved in the N cycle occur between the atmosphere, soil and water systems, and biomass. The main transformations occur in the soil

environment, are complex and can be done by both biological and non-biologically, such as nitrogen fixation, mineralization, immobilization, ammonification, nitrification, and denitrification (Delwiche 1970).

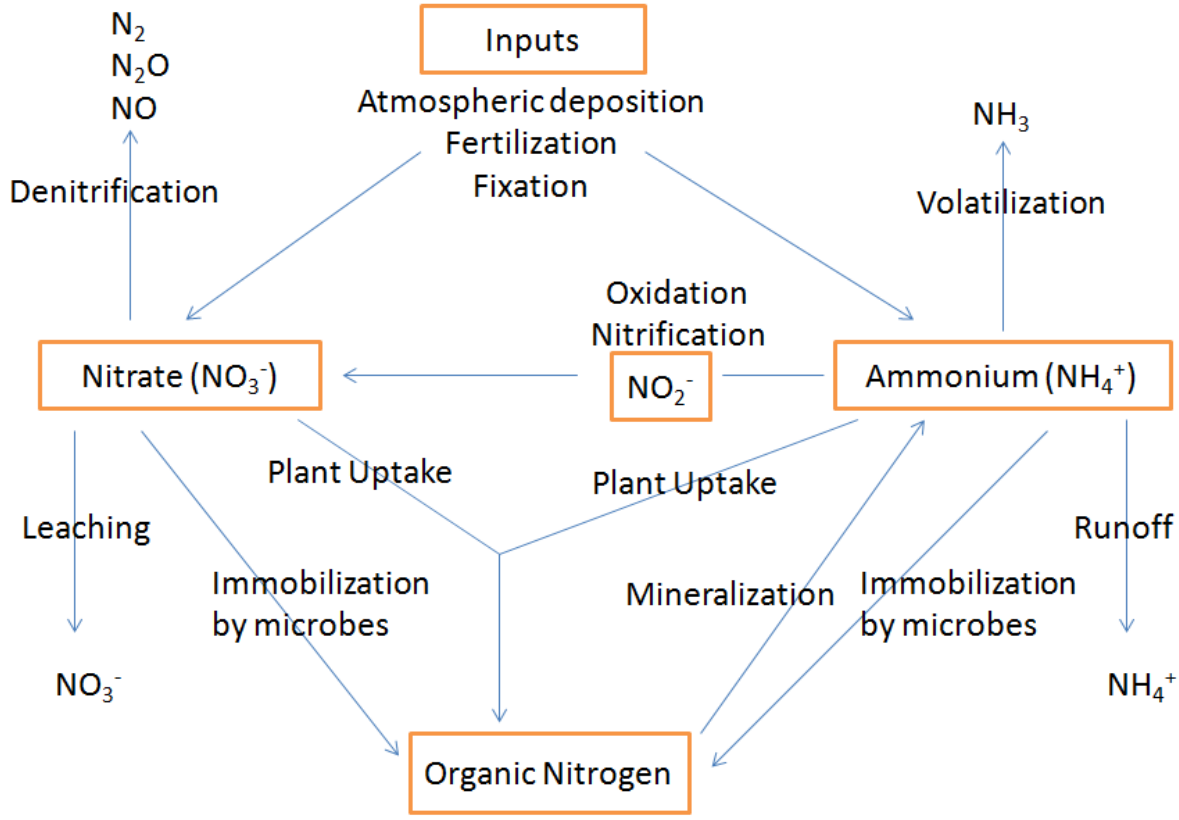


Figure 2.1 Nitrogen cycle in the ecosystem.

Nitrogen fixation occurs naturally by means of lightning. Lightning events produce enough energy to break the bonds in atmospheric N_2 molecules, causing the elemental N liberated to react with oxygen in the air forming nitrogen oxides. Nitrogen oxides then dissolve in rain water, producing nitrates and are carried to the soil. Nitrogen fixation can also occur synthetically. The most common method is the Haber–Bosch process, which requires high pressures and high temperatures, in conjunction with catalytic processes, to form ammonia used as a fertilizer. Ammonia can also be further processed to urea and

ammonium nitrate (Galloway 1998). Nitrogen fixation also occurs by biological conversion of nitrogen. Biological nitrogen fixation is carried out by free-living or symbiotic bacteria mostly in soil. For instance, a nitrogen fixing bacteria in the rhizobia group invades the roots of legumes to form nodules. The bacteria provide the plant with usable nitrogen, while the plant supplies the bacteria with carbons for their need.

Immobilization is a process that incorporates inorganic N, such as NH_4^+ and NO_3^-, into complex organic biomolecules that are unusable to plants. Mineralization is the inverse process of immobilization. It is a process that converts organic N into inorganic N, which is available to plants (Korsaeth et al. 2003). Nitrate (NO_3^-) is the most common nitrogen source available to higher plants (Druart et al. 2000). Nitrogen in this form is mobile and leachable because it carries a negative charge and is not adsorbed onto soil particles. Ammonium (NH_4^+) is also a preferred nitrogen source for plants. Plants use less energy for assimilation in this form when compared to NO_3^-. Ammonium carries a positive charge and is adsorbed onto soil particles. In this chemical form, leaching of nitrogen does not occur. Nitrogen in the ammonium form can be further converted to the nitrate form by soil bacteria (Douglas 1998). Ammonification is a part of mineralization (Schimel and Bennett 2004). Organic N exists in soil organic matter, crop residues, and manure. Soil microbes break down these complex organic biomolecules into ammonium, and this process is referred to as ammonification. Nitrification is the process whereby ammonium is oxidized to nitrites (NO_2^-) by some soil bacteria, such as the *Nitrosomonas* species, followed by the oxidation of the nitrites into nitrates by other bacterial species such as *Nitrobacter* (Schimel and Bennett 2004). Denitrification is the reduction of

nitrates back into nitrogen containing gases, N_2O or N_2 by another group of soil bacteria, such as *Pseudomonas*, hence completing the nitrogen cycle (Brady and Weil 2002).

2.4 Legume Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) is an efficient and renewable source of nitrogen for agriculture. It is a process in which atmospheric nitrogen is reduced to ammonia catalyzed by nitrogenase by a specialized group of microorganisms, such as the symbiotic *Rhizobium* and *Sinorhizobium*, or free-living soil bacteria, such as *Azospirillum* and *Azotobacter* (Wagner 2011). Research into BNF has been focused on the rhizobium-legume symbiosis because this association plays an important role in the N cycle (Mohammadi et al. 2012). This kind of symbiosis exists in different types of legumes, such as forage legumes, grain legumes, and some leguminous trees (Liu et al. 2011).

The complex process of legume BNF is affected by climatic factors, such as temperature and light, by soil conditions such as soil moisture, N concentrations, root zone pH, and content of necessary elements, and by biotic factors such as the absence of the required rhizobia species, plant genetic variation in N fixation ability, and pest damage (Al-Falih 2002). Any of these factors can negatively affect either rhizobial growth or that of the host plant itself and therefore can have a significant impact on symbiotic N fixation (Mohammadi et al. 2012).

2.5 Plant Species Impact on Ecosystem Nitrogen Cycling

Plants are an important part of nitrogen cycle because they take up atmospheric nitrogen and produce biomass, which then decomposes and releases nitrogen when they

die and decay. Plants can affect either nitrogen inputs (atmospheric deposition, animal movement, and nitrogen fixation) or losses (fire, animal movement, leaching, and denitrification) (Figure 2. 2). Some species, such as legumes and alder trees, have the ability to form a symbiotic relationship with rhzobia, and thus fix atmospheric nitrogen. Some species, for instance, the free-living *Azotobacter*, have effects on asymbiotic nitrogen fixation in the soil. In addition, the canopy architecture of plants also affects atmospheric deposition rates (Knops et al. 2002). Plant root exudates are important sources of carbon, which is a limitation for soil microbes. The carbon supplied by plants can determine the rate of net nitrogen mineralization, and consequently affect the production of nitrate. Much of the nitrogen lost in the ecosystem is from nitrate leaching. Thereby plants affect the nitrogen losses (Schmidt et al. 1997). Nitrogen fluxes in ecosystems are influenced by the fire regime. Fire decreases the biomass of the vegetation. This reduces water and nitrogen uptake, and results in an increase in soil water and nitrogen. As a result, more nitrates can be lost through leaching (Bustamante et al. 2006). Moving animals can indirectly increase nitrogen input into an ecosystem. Herbivores can slow down nitrogen cycling by eating plants such as legumes, resulting in the decrease of nitrogen pools by reducing legume nitrogen fixation (Ritchie et al. 1998). However, herbivores can also speed up nitrogen cycling by promoting plant growth and providing a more favorable environment for microbial decomposers. The waste of herbivores is also an available source of nitrogen for plants (Knops et al. 2002).

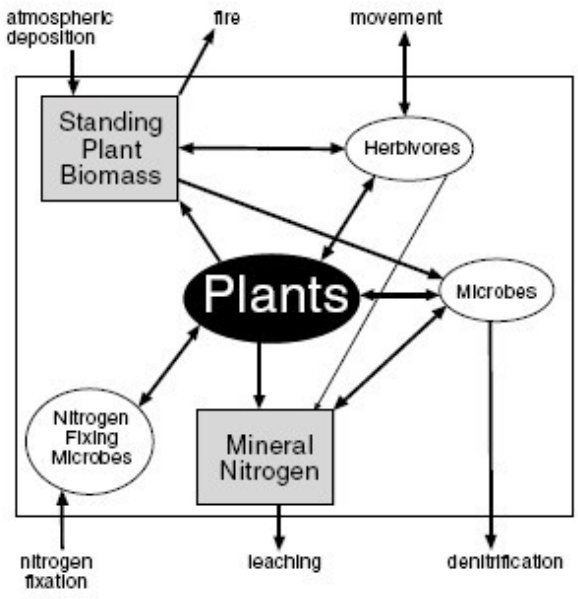


Figure 2.2 The major pathways of nitrogen inputs and losses (Knops et al. 2002).

2.6 Alfalfa's Contribution to the Cycling of Nitrogen in Agricultural Systems

Because of the increasing cost and negative impacts of synthetic nitrogen fertilizers on the environment, alfalfa, as well as other legumes, play an important role in global crop production. Alfalfa forage contains about 17.5% crude protein and 2.8% nitrogen. Approximately 76.5% of the total nitrogen in the herbage is derived via symbiotic fixation (Peterson and Russele 1991). Vance (1998) reported that alfalfa can fix up to 180 kg nitrogen per hectare per season. Alfalfa forms a deep and extensive root system and it has the capacity to absorb water and nutrients from the deep soil. This root system is very efficient in intercepting residual soil nitrogen. Biological nitrogen fixation by alfalfa is attributed to an association with nitrogen fixing bacteria, rhizobia. The total nitrogen content of soil increases during alfalfa growth. It has been suggested that about 56 kg of nitrogen per hectare is added to the soil each year (Peterson and Russele 1991). The nitrogen fixed by alfalfa is also available to subsequent crops in the rotation, as crop

residues break down (McNeil 2010). Therefore nitrogen fixation by alfalfa provides a significant source of nitrogen to the agri-ecosystem.

2.7 Rhizobia

Rhizobia are soil bacteria that have the ability to fix nitrogen after establishing symbiotic association with root nodules of legumes (Figure 2.3). Rhizobia are gram negative, motile, non-sporulating rods (Long 1996). They are now known to be phylogenetically diverse and include members of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Azorhizobium* (Mulder et al. 2005). Most of these bacterial species are in the family Rhizobiacea. The genus *Rhizobium* was the first named, and it currently consists of 22 species. The genus *Sinorhizobium* was first described in 1988 (Weir 2012). The *Rhizobium*-Legume symbiosis is characterized by a high level of host specificity. The host range of rhizobia may be narrow, for instance, the species of bacteria *Sinorhizobium meliloti* that forms nodules with alfalfa will not nodulate dry bean or soybean (Gough 2003). The host range could be broad as well, for example, *Rhizobium* sp. NGR 234 forms nodules with over 35 genera of legumes (Mulder et al. 2005). Nodule shape varies from spherical to elongate.

Figure 2.3 Nodules on alfalfa roots caused by *Sinorhizobium meliloti*.

The rhizobia that nodulate *Medicago, Melilotus*, and *Trigonella* species, are currently in the genus *Sinorhizobium* which contains two sister species *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*; Garau et al. 2005) and *Sinorhizobiun medicae* (Barran et al. 2002; Bailly et al. 2006). *Sinorhizobium meliloti* strain 1021 is one of the most well-studied and best characterized rhizobia. Its genome consists of three large replicons: a chromosome and two megaplasmids. These three replicons have also been sequenced (Capela et al. 2001).

2.8 *Rhizobium*-Legume Symbiosis

Legumes interact with rhizobia that convert atmospheric nitrogen into forms that are assimilated by plants. The interaction is an endosymbiosis because rhizobia enter legume cells (Cooper 2007). The host plant requires that rhizobia produce signal molecules, Nod

factors, to identify themselves, in order to prevent pathogens from entering by similar mechanisms (Smit 2007). Once rhizobia enter the root of the host plant, cortical cells previously differentiated have to be reactivated and enter the cell cycle so that a nodule primordium is formed. Release of rhizobia into primordium cells results in their differentiation into a nodule (Gage 2002). The nodules can be classified into two types, indeterminate nodules (*S. meliloti* and *M. truncatula*) and determinate nodules (*Mesorhizobium loti* and *Lotus japonicus*; Jones et al. 2007). Indeterminate nodules are cylindrical and elongated due to a persistent apical meristem. Nodule organogenesis begins with cell division in the inner cortex and pericycle of the root. The mature indeterminate nodule consists of several zones: I, meristem; II, infection; II-III, interzone; III, nitrogen fixation; IV, senescence (Figure 2.4). Determinate nodules are relatively spherical, do not have the persistent meristem and the cell division begins in the outer cortex (Mulder et al. 2005). Nodulation is generally initiated only in a susceptible region of the root, behind the root tips. With the development of nodules, the susceptible root region loses susceptibility. As a result the number of nodules going to be formed is controlled.

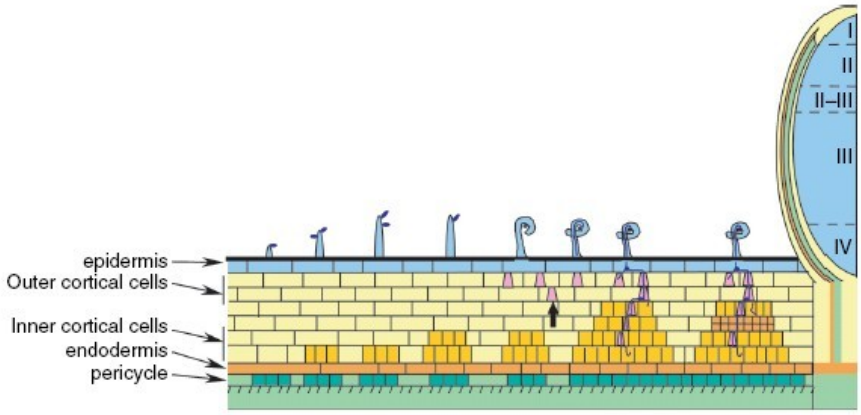


Figure 2.4 The development of an indeterminate nodule. The arrow shows the formation of pre-infection threads (Mulder et al. 2005).

The first step of the nodulation is signal exchange, which allows the rhzobia to enter the plant root hair cells. The infection process and the induction of cortical cell divisions are caused by Nod factors secreted by rhizobia when they colonize the root of the host plant (Smit 2007). Nod factors are lipochitoologosaccharide (LCOs) compounds produced by rhizobia. This process is signaled by flavonoids which are aromatic compounds (2-phenyl-1, 4-benzopyrone derivatives) secreted in the root exudates of legumes. They are the first signals to be exchanged during symbiosis (Figure 2.5a) (Jones et al. 2007). Flavonoids bind to bacterial NodD proteins, which are transcriptional regulators which in turn induce the transcription of other rhizobial genes (Perret et al. 2000). Among these induced rhizobial genes there are several *nod* genes encoding enzymes required for the production of Nod factors, which initiate several responses required for bacterial invasion of the plant host (Oldroyd and Downie 2004). The first response to Nod factor is an increase of calcium in root hairs, followed by calcium spiking and the modification to the root hair cytoskeleton. Then Nod factors cause the curling of the root hairs, which traps rhizobia within colonized curled root hairs (CCRH) (Figure 2.5a). At the same time, Nod factors stimulate root cortex cells to reinitiate mitosis to form the nodule primordium and induce cells that will receive the rhizobia (Gage 2004).

Before rhizobia can fix nitrogen they must be internalized by host cells in the root cortex (Perret et al. 2000). The bacteria penetrate host tissue by newly formed tubular structures known as infection threads. These structures are formed by trapped bacteria at the root hair tip. They produce both Nod factors and a symbiotically active

expolysaccharides to induce the ingrowth of the root hair cell membrane, which results in bacterial invasion (Figure 2.5b) (Gage 2004; Jones et al. 2007).

Once rhizobia enter the host root, they are internalized by cortical cells and a niche is formed within those cells. Each bacterial cell is endocytosed by a target cell in an endocytic membrane, and this entire unit is called symbiosome (Brewin 2004). Within this unit, the internalized bacteria then develop into bacteroids, a differentiated form that is capable of nitrogen fixation (Figure 2.5c). This process requires a constant carbon supply to provide metabolites and energy (Jones et al. 2007). Once the differentiation program is completed, bacteria synthesize enzymes of the nitrogenase complex and begin fixing nitrogen. Since the nitrogenase is oxygen-sensitive, the presence of oxygen in the symbiosomes would reduce the activity of the enzyme. Legumes produce leghemoglobin, which shows red color in nodules, to carry away free oxygen in the cytoplasm of infected plant cells to ensure the proper function of the enzyme activity (Fischer 1994).

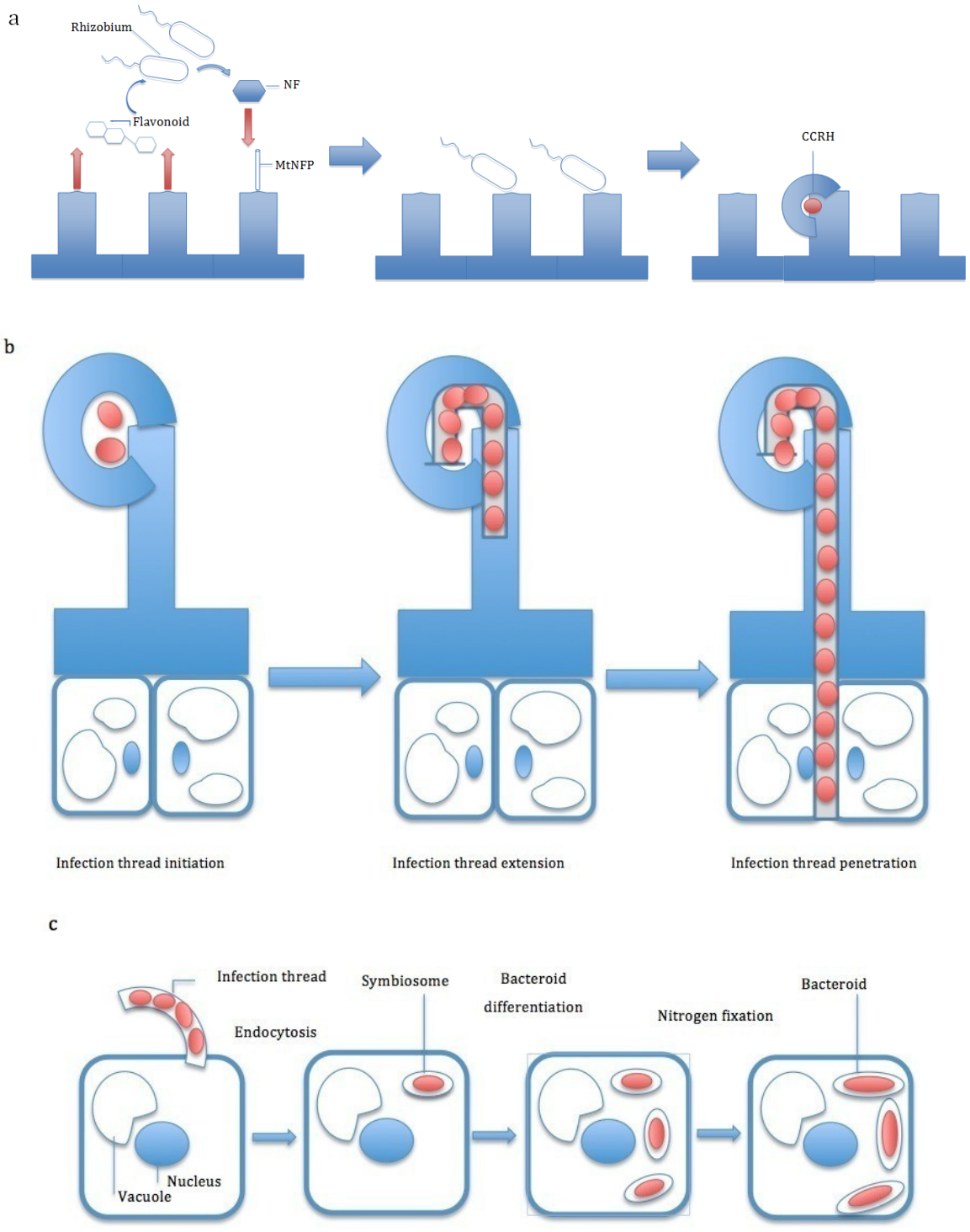


Figure 2.5 Rhizobium-legume symbiosis: (a) the initial signals exchange between *Sinorhizobium meliloti* and *Medicago truncatula*, Nod factors cause plant cortical cell division and root hair curling, (b) root hair invasion by *Sinorhizobium meliloti*, and (c) endocytosis of bacteria and bacteroid differentiation. The figures are modified based on Jones et al. 2007.

2.9 Factors Affecting Nodulation

2.9.1 *nod* Genes Expression

The *nod* genes are induced by signal molecules, usually flavonoids in the root exudates. They encode proteins that are involved in the production of the Nod factors (Mulder et al. 2005). The early stages of alfalfa nodule formation include bacterial stimulation of cortical cell divisions and bacterial invasion of the host root hairs (Cooper 2007). At least three sets of *S. meliloti nod* genes are involved in these early stages of nodule formation. One set is *nod*ABC, which present in all *Sinorhizobium* speicies. The second set of nodulation genes in *S. meliloti* includes *nod*FE, *nod*H, *nod*G, *nod*PQ and others. They are not necessarily present in all *Sinorhizobium* speicies and these genes affect the rate and frequency of nodule formation and influence the host range of the bacteria. The third set of nodulation genes, *exo* genes, is also required for normal nodule development. Most *exo* gene mutations of *S. meliloti* prevent normal bacterial invasion of the plant root (Finan et al. 1985). The genes of the *nod*ABC operon encode the proteins, which are essential to make the core structure of Nod factors. Other *nod* genes make modifications to Nod factors that impart host specificity, including the addition of fucosyl, sulphuryl, acetyl, methyl, carbamoyl, and arabinosyl residues. They also introduce differences in the acyl chain (Perret et al. 2000). A family of regulatory genes, *nod*D, has been identified in all *Sinorhizobium* species. Expression of *nod*ABC, *nod*FE, and *nod*H is induced by exudates of legume roots; this activation requires a *nod*D gene product (Mulligan and Long 1989; Long 1996). There are three *nod*D genes in *S. meliloti*, designated *nod*D1, *nod*D2, and *nod*D3 (Honma and Ausubel 1987). *nod*D3 requires a second gene, *syr*M (symbiotic regulator), for activating function. *syr*M can affect both the

expression of the *nod* genes and the expression or activity of the *exo* genes required for invasion of the plant roots (Mulligan and Long 1989).

2.9.2. Nod Factors

During early stages of the legume-rhizobia interaction, nodulation (Nod) factors are produced by rhizobia. Nod factors are lipochitooligosaccharides (LCOs). The first structural formula of a Nod factor from *S. meliloti* was reported in 1990 (Lerouge et al. 1990). They are composed of a β-1, 4-linked N-acetyl-D-glucosamine backbone with various functional group substitutions at the terminal or non-terminal residues (there usually 4 or 5 residues in total) (Long 1996). Nod factors are N-acylated at the non-reducing terminal residues. Different Nod factors contain different chemical substitutions on the sugar residues and /or variations in the structure of the acyl chains (Badri et al. 2009). For instance, the major Nod factor synthesized by *S. meliloti* consists of tetra-N-accetylglucosamine, acylated with a C16:2Δ2, 9 fatty acid, *O*-acetylated on C6 of the terminal non-reducing sugar, and *O*-sulfated on C6 of the reducing sugar (NodSm-IV, Ac, S, C16:2) (Mulder et al. 2006). Nod factors are essential signals in nodulation and without nod factors rhizobia cannot enter legume roots (Relic et al. 1994). The Nod factors are key determinants of host specificity, infection and nodule organogenesis, which stimulate physiological action at very low concentrations (10^{-12} - 10^{-9} M) (Dénarié et al. 1996). Nod factors initiate several symbiotic responses in the epidermal, cortical and pericycle cells of the host plants during nodulation. In epidermal cells of the host plant, Nod factors initiate biochemical responses including ion fluxes, membrane depolarization and calcium spiking. Nod factors also stimulate morphological changes

including root hair swelling, root hair branching and root hair curling. For legumes producing indeterminate nodules, Nod factors initiate cortical cell divisions in the inner cortex and pericycle (Jones et al. 2007). Nod factors are recognized by a specific class of receptor kinases called Lysin motifs (LysM) on the plant cell membranes. The *M. truncatula* Nod factor perception (MtNFP) is a member of the LysM family of receptors. NFP is the sole gene identified in *M. truncatula*, which is required for root hair curling and the induction of transcriptional changes in response to Nod factor (Mulder et al. 2006). Many rhizobia produce more than one type of Nod factor. Purified Nod factor can initiate several signaling responses without the presence of rhizobia (Barnett and Fisher 2006).

2.9.3 Phytohormones

Plant growth hormones, auxin, cytokinin, gibberellin, and ABA, are present in the nodules. The concentrations of these hormones are higher than in the uninfected root tissues. Thus it appears plant growth hormones play a role in the nodulation. Ethylene is known as a potent inhibitor of nodulation, and acts at the early stages of nodulation (Hirsch and Fang 1994). Ethylene also plays a role in controlling the nodule number (Mulder et al. 2005). In *M. truncatula*, increasing the concentration of ethylene precursors 1-aminocyclopropane-1-carboxylic acid (ACC) decreases both the nodule number and the initiation of infection threads, while increasing the concentration of aminoethoxyvinylglycine (AVG), which is an ethylene synthesis inhibitor, increasing the number of nodules and infection threads (Oldroyd et al. 2001). Inhibition of polar auxin transport is an early step in nodulation and is controlled by Nod factors. It is suggested

that Nod factors cause an increase in both auxins and cytokinins in the progenitor cells of nodules. Auxin and cytokinin interact in nodule organogenesis to initiate cell division and the formation of nodule primordia (Mulder et al. 2005). Due to an exogenous auxin transport inhibitor, the ratio of these two hormones could be unbalanced. An increase in the cytokinin/auxin ratio is suggested by the observation that cytokinins applied to roots induced divisions in cortical cells (Relid et al. 1993). Similarly, *S. meliloti* transconjugants carrying the tzs (trans-zeatin secretion) gene of *Agrobacterium tumefaciens*, and presumably secreting cytokinin, induced bacteria-free nodules on alfalfa (Cooper and Long 1994).

There are at least two explanations regarding the relationship between Nod factor and nodule organogenesis. One suggests that the Nod factor acts as a hormone to activate cell divisions and the formation of nodule primordia. The second hypothesis suggests that there is a change in the endogenous hormone balance caused by Nod factor treatment (Hirsch and Fang 1994).

2.9.4 Root Exudates and Nodulation

Plants release a large variety of organic and inorganic compounds in the root exudates, which are involved in complex communication processes in the rhizosphere (Seigler 1998). Root exudates influence the soil microbial community, modify soil properties, inhibit the growth of neighboring plants and enhance beneficial symbiotic associations (Long 1996; Sanon et al. 2009). The composition of root exudates include sugars, polysaccharides, amino acids, aliphatic acids, aromatic acids, fatty acids, sterols, phenolics, enzymes, vitamins, plant growth regulators and other secondary metabolites

(Bertin et al. 2003; Prithiviraj et al. 2007). Many of those secondary metabolites have been shown to be of ecological significance because they are toxic to insects and plant pathogens, or because they act as signalling molecules, in the air as well as in the rhizosphere (Bouwmeester et al. 2007). Among these compounds, flavonoids are known to act as key signaling molecules for plant microbe interactions in the soil. Flavonoids are synthesized by the phenylpropanoid pathway and the acetate-malonate pathway. They act as inducers of *nod* genes of rhizobia, which are essential for the synthesis of Nod factors, when released from seed coats or roots of legumes (Cooper 2007). About 30 *nod* gene-inducing flavonoids have been isolated from nine legume genera. They are either glycones or aglycones from flavonoid subclasses including chalcones, flavones, flavanones, isoflavones and coumestans (Cooper 2004). In the legume-rhizobia symbiosis, flavonoids attract rhizobial bacteria and act as specific inducers of rhizobial *nod* genes, which are involved in the synthesis of Nod factors. In roots of legumes Nod factors induce the synthesis of flavonoids and eventually in the secretion of more flavonoids by the root, which further stimulate the production of Nod factors by the bacteria (Steinkellner et al. 2007). Transcription of *nod* genes is required for Nod factor synthesis and is usually mediated by NodD proteins, which are members of the LysR family of transcriptional regulators. Flavonoids bind to NodD in the promoter site therefore initiate gene transcription (Jones et al. 2007). The first molecule showing nod gene-inducing activity in *S. meliloti* was isolated from alfalfa seeds and identified as 3', 4', 5, 7-tetrahydroxyflavone. This compound is known as luteolin (Peters et al. 1986). The dominant flavonoid released from alfalfa seeds was identified as quercetin-3-O-galactoside. However, this flavonoid does not induce *nod* genes. Low concentrations (1-

10 mM) of this compound and other flavonoids, including luteolin-7-O-glucoside, aglycones, quercetin, and luteolin, can increase the growth rate of *S. meliloti* (Hartwig et al. 1991).

2.9.5 Environmental Factors

Environmental stresses are generally limiting factors to symbiotic nitrogen fixation. If the soil nutrients and pH are adequate, the major factors affecting nitrogen fixation efficiency are temperature, light, and moisture. Other factors that affect nitrogen fixation include high soil nitrogen, phosphorus deficiency, high soil salinity, and soil acidity (Al-Falih 2002).

Legumes need more energy to fix N biologically than take up N from soil. Therefore the presence of mineral N in the soil inhibits legume nodulation, nodule establishment and nitrogenase activity. However, in some circumstances, certain concentrations of N in the soil, normally less than 4 mM for ammonium and less than 2 mM for nitrate, stimulates nodule establishment and N fixation. This low concentration of N is termed "starter N" (Liu et al. 2011).

Nodulation is obviously affected at low pH in alfalfa (Jo et al. 1980). Under soil acidity conditions, nodules formed by the rhizobia may not have the ability to fix nitrogen or the fixation rate may be inadequate (Mohammadi et al. 2012). *S. meliloti* usually does not grow below pH 5.3. Brockwell et al. (1991) reported a nearly 10^{-3} decrease in the number of *S. meliloti* in soils with a pH<6 compared to those with a pH>7.0. Acid soils often contain low concentrations of phosphate and Ca, as well as high concentration of heavy metals that adversely affect the process of nodulation. At low pH, even the

rhizobial numbers are high, nodulation of alfalfa is reduced (Al-Falih 2002) suggest that acidity reduce the efficiency of nodulation.

Many soils are rich in salt and have high pH values. The predominant salts in the soil are sulphates and chlorides of sodium, calcium, potassium, and magnesium. These areas are often considered undesirable for legumes (Al-Falih 2002). Alfalfa is a salt tolerant legume, however, the tolerance varies with different salts. For instance, potassium chloride has a more negative effect on alfalfa nodulation than magnesium chloride (Eaglesham and Ayanaba 1984).

Soil temperature is one of controlling factors for nodulation. Rhizobia species show varying susceptibility to high temperatures. The optimum temperatures for rhizobia growth among different strains and species vary between 25-35 ^{o}C. The maximum temperatures for N fixation differ among species usually from 35 to 40 ^{o}C and minimum temperatures from 2 to 10 ^{o}C (Liu et al. 2011). It appears that every legume and rhizobium combination has an optimum temperature relationship (Mohammadi et al. 2012).

Similar to soil temperature, soil moisture controls N fixation through nodulation and gas permeability (Liu et al. 2011).Under drought conditions, the number of rhizobia in soils is reduced, and both nitrogen fixation and respiration of nodules are inhibited. Excessive moisture in the soil prevents the development of root hair and sites of nodulation, and influence on the oxygen diffusion in the plant root system (Al-Falih 2002).

2.10 Seaweed

There are about 9,000 species of marine macro-algae, included in the brown, green, and red algae (Phaeophyta, Chlorophyta, and Rhodophyta, respectively) groups, based on their pigments, such as chlorophylls, carotenoids and phycobilins (Khan et al. 2009). Seaweeds are one of the most important marine resources and have long been used as human food, fodder, manure, and raw materials for some industries (Sathya et al. 2010). Some seaweed products exhibit crop growth-stimulating properties. Application of seaweed products is now becoming an accepted practice in horticulture, because of its beneficial effects (Fornes et al. 2002). Seaweed application increases plant growth, yield and quality, at least in part as the extract contains carbohydrates, plant hormones (Abscisic acid, Auxins, Cytokinins, and Gibberellins), hormone-like plant growth regulators (Betaines, Brassinosterols, Jasmonates, Polyamines, Salicylates, and Signal peptides), trace elements (Fe, Cu, Zn, Co, Mo, Mn, and Ni), vitamins and amino acids (Challen and Hemingway 1965; Khan et al. 2009; Craigie 2010). It has been shown that seaweed components affect cellular metabolism in plants. Seaweed extracts also have been reported to increase the uptake of nutrient elements from the soil, and enhance plant resistance to pests and disease (Beryln and Russo1990; Verkleij 1992). There are a number of commercial seaweed products that can be used in agriculture and horticulture, which are presented in Table 2.1.

Table 2.1. Commercial seaweed products used in agriculture and horticulture (Khan et al. 2009).

Product name	Seaweed name	Company	Application
Acadian®	*Ascophyllum nodosum*	Acadian Agritech	Plant growth stimulant
Acid Buf	*Lithothamnium calcareum*	Chance & Hunt Limited	Animal feed
Agri-Gro Ultra	*Ascophyllum nodosum*	Agri Gro Marketing Inc.	Plant growth stimulant
AgroKelp	*Macrocystis pyrifera*	Algas y Bioderivados Marinos, S.A. de C.V.	Plant growth stimulant
Alg-A-Mic	*Ascophyllum nodosum*	BioBizz Worldwide N.V.	Plant growth stimulant
Bio-Genesis™ High Tide™	*Ascophyllum nodosum*	Green Air Products, Inc.	Plant growth stimulant
Biovita	*Ascophyllum nodosum*	PI Industries Ltd	Plant growth stimulant
Emerald RMA	Red marine algae	Dolphin Sea Vegetable Company	Health product
Espoma	*Ascophyllum nodosum*	The Espoma Company	Plant growth stimulant
Fartum®	Unspecified	Inversiones Patagonia S.A.	Biofertilizer
Guarantee®	*Ascophyllum nodosum*	MaineStream Organics	Plant growth stimulant
Kelp Meal	*Ascophyllum nodosum*	Acadian Seaplants Ltd	Plant growth stimulant
Kelpak	*Ecklonia maxima*	BASF	Plant growth stimulant
Kelpro	*Ascophyllum nodosum*	Tecniprocesos Biologicos, S.A. de C.V.	Plant growth stimulant
Kelprosoil	*Ascophyllum nodosum*	Productos del Pacifico, S.A. deC.V.	Plant growth stimulant
Maxicrop	*Ascophyllum nodosum*	Maxicrop USA, Inc.	Plant growth stimulant
Nitrozime	*Ascophyllum nodosum*	Hydrodynamics International Inc.	Plant growth stimulant
Profert®	*Durvillea antarctica*	BASF	Plant biostimulant
Sea Winner	Unspecified	China Ocean University Product Development Co., Ltd	Plant biostimulant
Seanure	Unspecified	Farmura Ltd.	Plant growth stimulant
Seasol®	*Durvillea potatorum*	Seasol International Pty Ltd	Plant growth stimulant
Soluble Seaweed Extract	*Ascophyllum nodosum*	Technaflora Plant Products, LTD	Plant growth stimulant
Stimplex®	*Ascophyllum nodosum*	Acadian Agritech	Plant growth stimulant
Synergy	*Ascophyllum nodosum*	Green Air Products, Inc.	Plant growth stimulant
Tasco®	*Ascophyllum nodosum*	Acadian Agritech	Animal feed

Among brown seaweeds, *Ascophyllum nodosum* (L.) Le Jol. is the most researched (Khan et al. 2008; Khan et al. 2012). It is distributed largely in the cold waters of the North Atlantic Ocean extending from eastern Canada to parts of northern Europe (Cardozo et al. 2007). *A. nodosum* contains polysaccharides such as laminaran, fucoidan, and alginate (Lane et al. 2006). Among these three polysaccharides, laminaran and fucoidan exhibit a wide range of biological activities. Laminaran stimulates natural defense responses in plants (Fritig et al. 1998). Cytokinins, auxins and auxin-like compounds have been detected in *A. nodosum* extract. Extracts of *A. nodosum* (ANE) had as much as 50 mg IAA per gram of dry extract (Kingman and Moore 1982). ANE contains various betaines and betaine-like compounds, such as Glycine betaine, γ-amino butyric acid betaine, δ-aminovaleric acid betaine and laminine (Blunden et al. 1986; Khan

et al. 2009). They are N-methylated compounds, which are cytoplasmic osmoticants physiologically (Craigie 2010). Betaines protect against osmotic, drought, frost, high salinity and high temperature stresses. It is also suggested that betaines may work as a nitrogen source when provided in low concentrations (Khan et al. 2009). Foliar and soil application of ANE to plants stimulates shoot growth and branching (Temple and Bomke 1989), enhances endogenous antioxidant activity (Zhang and Ervin 2004; Di et al. 2011), increases root growth and crop yield, and resistance to biotic and abiotic stresses (Rayorath et al. 2008b, 2009; Khan et al. 2009). The organic and inorganic composition and elemental analysis of the commercial ANE is presented in Table 2.2

Table 2.2. The total organic and inorganic composition and elemental analysis of *Ascophyllum nodosum* extract (ANE) (Acadian Seaplants Limited, technical information).

Composition	ANE (dry powder)
Organic Matter	**45-55** %
Alginic acid	12-16 %
Fucose Polymers	13-17 %
Mannitol	4-6 %
Amino Acids	4-6 %
Other Organic compounds	**10-12 %**
Ash	**45-55 %**
Nitrogen	0.8-1.5 %
Phosphorus	0.5-1.0 %
Potassium	14-18 %
Calcium	0.3-0.6 %
Iron	75-250 ppm
Magnesium	0.2-0.5 %
Manganese	8-12 ppm
Sodium	3.0-5.0 %
Sulfur	1.0-2.0 %
Zinc	10-25 ppm

Chapter 3.0 Hypothesis and Objectives

3.1 General Hypothesis

Application of seaweed extract to alfalfa plants will increase the nitrogen fixation potential of alfalfa by altering physiological and molecular processes both under greenhouse and field conditions.

3.2 Objectives

1) To evaluate application of seaweed extracts on the development of nitrogen fixing root nodules, vigor and yield of alfalfa under greenhouse and field conditions.

2) To investigate the mechanism(s) by which seaweed extracts improve the *rhizobium*-legume symbiosis.

Chapter 4.0 Materials and Methods

Seeds of *Medicago sativa* L. (Alfalfa) cv. Caribou were obtained from the Department of Plant and Animal Sciences, Nova Scotia Agricultural College, Truro, Nova Scotia. *Sinorhizobium meliloti* strain 1021 was a kind gift from Dr. Trevor Charles, Department of Biology, University of Waterloo, Ontario, Canada. The bacterial culture of NodC::LacZ construct of *S.meliloti* (JM57) was a kind gift from Sharon R. Long, Department of Biological Sciences, Stanford University, Stanford, California, USA. The culture was maintained on tryptone/yeast extract (TY) media (Vincent 1970) containing 200 μg mL^{-1} streptomycin. The commercial inoculant of *S. meliloti* strain NRG-185-1 for the field experiments was purchased from the store TRA Cash & Carry (Truro, NS). Soluble extract of *Ascophyllum nodosum* (ANE) powder was provided by Acadian Seaplants Limited, Dartmouth, Nova Scotia, Canada. All chemicals (Table 4.1) needed for Long Ashton Standard Nutrient Solution (LANS) were purchased from Fisher Scientific (Ottawa, ON). All the other chemicals and reagents were of analytical grade and were purchased from Sigma Aldrich (Oakville, ON, Canada), unless otherwise stated.

Table 4.1. Concentrations of stock solutions for the Long Ashton Standard Nutrient Solution

Compound	Stock Conc'n (% w/v)	LANS Dilution (mL/L)	LANS 0.1 x Dilution (mL/L)	LANS-M SSEP 0.1 x Dilution (mL/L)	LANS-M pH8 0.1 x Dilution (mL/L)
KNO_3	2.02	20	2	2	2
$Ca(NO_3)_2$ $4H_2O$	4.72	20	2	0.4	0.4
$MgSO_4$ $7H_2O$	1.84	20	2	2	2
NaH_2PO_4 H_2O	1.84	10	1	1	0.15
Fe-citrate H_2O	0.25	5	0.5	0.5	0.5
$MnSO_4$ H_2O	0.034	5	0.5	0.5	0.5
$CuSO_4$ $5H_2O$	0.0125	1	0.1	0.1	0.1
$ZnSO_4$ $7H_2O$	0.058	1	0.1	0.1	0.1
H_3BO_3	0.186	1	0.1	0.1	0.1
Na_2MoO_4 $2H_2O$	0.0121	1	0.1	0.1	0.1
NaCl	0.585	1	0.1	0.1	0.1
$CoSO_4$ $7H_2O$	0.0053	1	0.1	0.1	0.1
KCl	6.24	-	-	2	2
KOH	4.72	-	-	2	2

4.1 Greenhouse Experiments

4.1.1 Plant Growth Assay

This experiment was carried out to study the effects of ANE on nodulation and growth of alfalfa plants under greenhouse conditions. Alfalfa seeds were planted at a 1.5

cm depth in plastic pots (10.2 cm diameter; 3 seeds per pot) containing sterile vermiculite. Pots were kept under greenhouse condition set at 22/18 °C day/night temperature, 16:8 photoperiod, light intensity 200-300 μmol $m^{-2}s^{-1}$, and a relative humidity of 75%. Plants were thinned to 1 seedling per pot after germination. Nitrogen free Hoagland's solution was applied one week after seeding, followed by application at every 3rd day at the rate of 100 mL per pot. Solutions of ANE were prepared by dissolving 0.1 g, 0.5 g, 1.0 g, or 2.0 g soluble powder in 1.0 L distilled water with continuous stirring for 10 min. The experimental plants were irrigated with various ANE concentrations at the rate of 50 mL per plant on day 10 after seeding, as described by Khan et al. (2008). No ANE (only water) was used as the control. Two days after the ANE treatments, the plants were inoculated with *S. meliloti* 1021 OD_{600} 0.1 cell suspension at the rate of 1 mL per plant.

Thirty two days after ANE treatment (six week old plants), plants were harvested for the observation of nodule formation and growth parameters. Plants were gently removed from vermiculite and washed with tap water. Total number of nodules and functional nodules (pink or red color due the presence of leghemoglobin) were counted. Root length was measured by WinRhizo (Regent Instruments Inc. Quebec, Canada) and leaf area was measured by WinFoliar (Regent Instruments Inc. Quebec, Canada). Roots and shoots were separately put into paper envelops and dried in an oven at 60 °C. Root dry weight and shoot dry weight were measured after 3 days of drying. The experiment was conducted following a randomized complete block design with 5 replications for each treatment and was repeated 3 times.

4.1.2 Seed Priming Assay

This experiment was conducted to study the effect of ANE on the growth of alfalfa under salinization conditions in the greenhouse. Seeds of alfalfa were soaked in different concentrations of ANE (0, 0.25, 0.5, and 1 g L^{-1}) for 12 h and were then held at room temperature until dry. Alfalfa seeds were then planted at a 1.5 cm depth in plastic pots (10.2 cm diameter; 2 seeds per pot) containing Pro-mix applied with 100 mM NaCl. They were thinned to 1 plant per pot after germination. Pots were placed under greenhouse conditions, as already described for the plant growth assay. One week after germination, plants were inoculated with *S. meliloti* 1021 OD_{600} 0.1 cell suspension (1 mL per plant). Application of nitrogen free Hoagland's solution was initiated one week after planting and then applied every 3 days at the rate of 100 mL per pot. Plants were grown for 3 weeks and then harvested for whole plant length measurement with a ruler. This experiment was conducted following a randomized complete block design with 5 replications for each treatment and was repeated 3 times.

4.1.3 Seed Pelleting Assay

This experiment was carried to study the effect of ANE on the early stages of alfalfa growth under greenhouse conditions. Seeds of alfalfa were pelleted with a thin layer of 5% carboxymethyl cellulose, as a sticking agent, and then treated with different concentrations of the mixture of powdered ANE and talc (25%, 50%, 75%, and 100% ANE) (Figure 4.1). They were planted 1.5 cm deep in plastic pots (10.2 cm diameter; 2 seeds per pot) containing Pro-mix. They were thinned to 1 plant per pot after germination. Pots were grown under the greenhouse conditions as already described for the plant

growth assay. Plants were harvested 5, 10, and 15 days after planting. They were gently removed from Pro-mix and washed with tap water. Root length was measured by WinRhizo and leaf area was measured by WinFoliar. This experiment was conducted following a randomized complete block design with 15 replications for each treatment and was repeated 3 times.

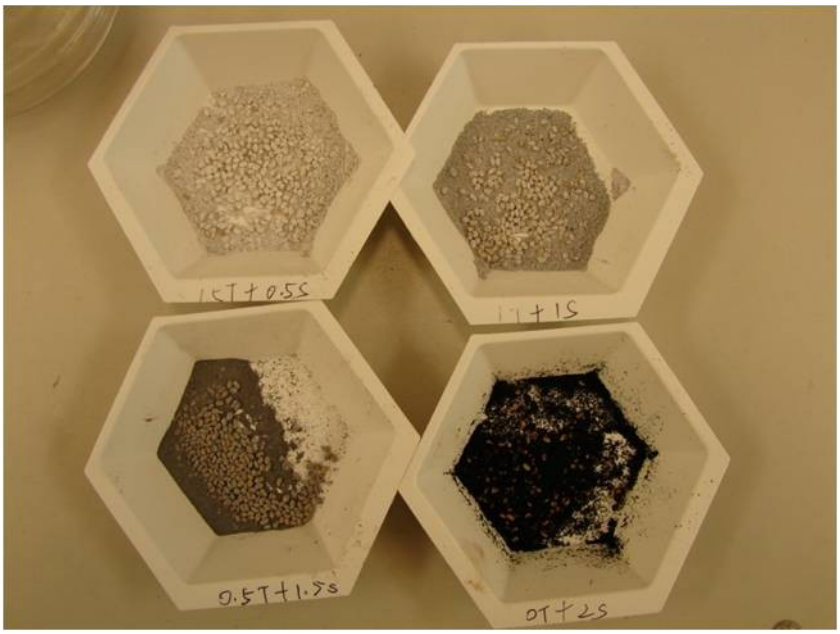


Figure 4.1 Alfalfa seeds pelleted with different concentrations of the mixture of powdered ANE and talc.

4.2 Laboratory Experiments

4.2.1 Root Hair Deformation Assay

The *in vitro* root hair deformation assay was conducted to investigate the ability of ANE to elicit root hair deformation in alfalfa roots. The root hair deformation assay described by Prithiviraj et al. (2000) was used. Alfalfa seeds were surface sterilized in 70% ethanol (EtOH) for 1 min and then shaken for 30 min at 100 rpm in 50 mL 2.6% (w/v) sodium hypochlorite containing 100 µL Tween-20. The seeds were then rinsed in sterile distilled water for 5 times. Seeds were planted on half-strength Murashige and Skoog

basal medium (pH 5.8), which contained 1% (w/v) sucrose and 0.8% (w/v) agar in Petri dishes (9 cm diameter), at a rate of 5 seeds per plate. Lateral roots of ten-day-old alfalfa seedlings were treated with 50 µL of various concentrations of ANE (0, 0.1, 0.5, 1, or 2 g L^{-1}) by root irrigation. ANE solutions were filter sterilized using a 0.22 µm SFCA syringe filter (Corning Inc. NY, USA). One day after the ANE treatments, the roots were inoculated with 20 µL*S. meliloti* 1021 OD_{600} 0.1 cell suspension per plant. Plates were kept in the dark at 20 ± 2 °C for incubation. Root hair deformation was observed 6, 12, and 24 h post inoculation. Roots of alfalfa were gently cut from plants using a sterilized blade and washed with sterile distilled water. They were placed on a microscope slide and number of deformed root hairs was observed under a light microscope. For this experiment, each petri dish (with 5 plants) is a replicate. The experiment was repeated 2 times with 3 replications per treatment.

4.2.2 β-Galactosidase Assay

S.meliloti JM57 is the rhizobial strain carrying the pRmM57 plasmid which has the *nodC-lacZ* fusion (Mulligan and Long 1985). Assay for β-Galactosidase activity was performed to investigate the effect of ANE on the translational fusion NodC::LacZ expression.

4.2.2.1. Qualification of the β-galactosidase Activity

Different inducer-responses can be measured indirectly by using *S.meliloti* JM57 *NodC* gene expression activity, based on the amount of β-galactosidase activity. Bacteria were grown on minimal medium (Hartwig et al. 1991) supplemented with 50 µg mL^{-1}

spectinomycin and 20 mg mL^{-1} X-Gal in Petri dishes (6 cm diameter). The X-Gal substrate indicated the expression of β-galactosidase activity due to *lacZ* gene activity, where X-Gal is cleaved by β-galactosidase producing 5-bromo-4-chloro-3-hydroxyindole and galactose. The former is then oxidized resulting in an insoluble blue pigment. Therefore, blue colonies with functional *lacZ* genes were visually detectable. Media were supplemented with different concentrations of ANE (0, 0.1, 0.3, or 0.5 g L^{-1}) and 10 μM luteolin in the medium was used as the positive control. All ANE were sterilized using sterile syringe filters. The plates were incubated with 50 μL of the culture and kept in the incubator at 28 ^{o}C. Observations were taken after one week.

4.2.2.2. Quantification of the β-galactosidase Activity

S. meliloti JM57 were grown in liquid minimal medium overnight at 30^{o}C. Approximately 20 to 50 μL of the overnight culture was added to the fresh minimal medium containing ANE 0, 0.1, 0.3, or 0.5 g L^{-1} or 10 μM luteolin used as the positive control. They were shaken until bacteria had grown to the mid- or late-log phase: 2-5 × 10^{7} cells mL^{-1} (OD_{600}=0.5 to 1.0) in minimal medium. The cells were centrifuged 5 min at 2500 rpm in a tabletop centrifuge. The cell pellets were then resuspended in an equal volume of Z buffer (Miller 1972) and placed on ice. Observations were recorded at 600 nm using a spectrophotometer (Shimadzu UV-1700, Shimadzu Corp., Tokyo). Samples (0.5 mL) were taken and mixed with 0.5 mL Z buffer; one drop of 0.1% SDS and 2 drops of chloroform were added, vortexed for 15 sec and equilibrated for 15 min in a 30 ^{o}C in a water bath. The samples were assayed at 30 °C for β-galactosidase using ortho-nitrophenylgalactoside (ONPG) substrate, as described by Miller (Miller 1972). Before

determining the optical density at 420 nm, the samples were centrifuged for 5 min at 2500 rpm. The β-galactosidase activity was measured as "Miller Units" calculated with the following equation:

$$1\ Miller\ Unit = \frac{1000\ \times\ (OD420 - 1.75\ \times OD550)}{t\ \times v\ \times OD600}$$

Where:

OD_{420} is the absorbance of the yellow o-nitrophenol,

OD_{550} is the scatter from cell debris, which, when multiplied by 1.75 approximates the scatter observed at 420 nm,

t = reaction time in minutes,

v = volume of culture assayed in milliliters,

OD_{600} reflects cell density.

This experiment was conducted 3 times with 3 replications per treatment.

4.3 Field Experiments

4.3.1 Site Description and Treatments

The field experiment was conducted in 2009 and 2010 to determine the effect of *A. nodosum* extract on nodulation and growth of alfalfa. The experiment was set up at two sites, including the AgriTech Park Field of the Nova Scotia Agricultural College (NSAC; Truro Trial), Truro, NS, and the Nappan Research Farm of Agricultural and Agri-Food Canada (AAFC; Nappan Trial), Nappan, NS. The native soil at the Truro trial site is a very rapidly draining sandy soil (Webb 1991). The climate conditions are cool, humid and temperate. The whole year average daily temperature was 6.1°C and annual precipitation was 1169 mm (averages calculated from 1971-2000; Environment Canada

2010). Meteorological conditions were recorded via Environment Canada's weather monitoring station in nearby Debert, Nova Scotia. The soil in for Nappan trial was a coarse loamy till (<18% clay), 50 – 80 cm deep with a 2-5% slope (Webb and Langille 1995), which had the following soil analysis: pH = 6.4, organic matter (OM) % = 3.1, total N% = 0.0657, P_2O_5 = 675 kg ha^{-1}, K_2O = 185 kg ha^{-1}. The climate conditions were cool and humid. The whole year average daily temperature was 5.8°C and annual precipitation was 1175 mm (averages calculated from 1971-2000; Environment Canada 2010). Meteorological conditions were recorded via Environment Canada's weather monitoring station on site at the Nappan Research Station, Agriculture and Agri-Food Canada. Fertilizer 3-15-6 Boron 03 was applied before planting at a rate of 290 kg ha^{-1} at the Nappan Trial on June 4th, 2009, and fertilizer application was done at the Truro Trial site on June 5th, 2009. These were the only dates fertilizer was applied during this experiment. The experiment was conducted following a 6 × 6 Latin Square Design with 6 treatments (LANS as control; ANE 0, 1, 2, 3, 4 g L^{-1}). The area of each plot was 1.5 × 6 m and two more plots were set up at each side as guard to avoid edge effects (Figure 4.2a). The Nappan Trail was seeded on June 4th, 2009, whereas the Truro Trial was seeded on June 5th, 2009. Both trials were seeded using a plot drill (Figure 4.2b). The seeding rate at both sites was 14 kg ha^{-1}. Hand weeding was used whenever weed control was required. Seeds of alfalfa were mixed with *S. meliloti* inoculant at a rate of 14.2 g inoculant per 22.7 kg seeds, before seeding. Treatments (25 L for each plot) were applied using a watering can for root irrigation (Figure 4.2c); these were applied once a month for three months. The first application of treatments was done on June 26th, 2009 and June 29th, 2009 for the Nappan and Truro trials, respectively.

Figure 4.2 Field site design and treatments: (a) 6 × 6 Latin Square Design experiment, (b) the plot drill used for seeding, and (c) watering cans used for ANE application as root irrigation.

4.3.2 Data Collection

Plants were harvested for yield determination during the establishment and post establishment years (2009 and 2010, respectively). A total of 10 plants from each plot were excavated using a shovel to about 15-20 cm depth before each harvest. These samples were thoroughly washed using tap water. Total numbers of functional nodules were counted based on 5 plants per plot. Total length of the tap root, secondary roots and root hairs were measured by WinRhizo based on 5 plants per plot. Total surface area of leaves was measured by WinFoliar based on 5 plants per plot. Roots and shoots were separated and put in paper bags and dried in a forced air drying oven at 60 ^{o}C for three days. They were then weighed based on 10 plants per plot. Because of severe weed

problems at the Truro Trial site, plants were not sampled for the observation of plant growth parameters. They were only harvested for the yield analysis. For the establishment year, the Nappan trial was first harvested on July 24th, 2009 and the Truro Trial was first harvested on July 29th, 2009; harvests were conducted with a Haldrup grass harvester F-55 (Kincaid Equipment Manufacturing, Haven, USA) (Figure 4.3). The yield of each plot was weighed and a small amount of leaves (2-3 g) from each plot were ground for the mineral element analysis. The second harvest was done on August 28th, 2009 for the Nappan Trial and September 3rd, 2009 for the Truro Trial. At the Truro site, for the second harvest, there was not enough forage to do a machine harvest, therefore a rectangular 0.25 m^2 quadrate was used for sampling. For the post establishment year, the Nappan trial was first harvested on July 19th, 2010 and the Truro Trial was first harvested on July 23rd, 2010 by haldrup. The second harvest was done on September 1st, 2010 for the Nappan Trial and September 3rd, 2010 for the Truro Trial.

Figure 4.3 Haldrup used for alfalfa harvesting.

4.3.3 Mineral Element Analysis

After drying, alfalfa leaves were ground to a fine powder by using a mortar and pestle. Concentrations of the following minerals nitrogen (N), sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), manganese (Mn), and Phosphorous (P) were determined at the Nova Scotia Agriculture Laboratory Services, Harlow Institute, Truro, N.S. by the AOAC method 968.08 (radial ICP).

4.4 Statistical Analysis

Experimental data were analyzed by one-way analysis of variance (ANOVA) and differences between the control and ANE-treated plates were analyzed by Tukey's Honestly Significant Differences (HSD) Test with $P \leq 0.05$ using CoStat statistical

software (CoHort Software, Monterey, CA).The medium was supplemented with 0 g L^{-1} ANE, which meant that they received an equal amount of sterilized distilled water in the extract treatment, and these were considered as control plants. Field data were analyzed using GenStat statistical software (Payne 2002) and the Standard Error of the Mean (SEM) for comparison of means was calculated at a probability level of $P < 0.05$. Polynomial contrasts were used to detect differences between the levels of treatments and an orthogonal contrast to evaluate the difference between the control and all other treatments. All the graphs were generated using SigmaPlot software version 12.0 (Systat Software, San Jose, CA).

Chapter 5.0 Results

5.1 Greenhouse Experiments

5.1.1 Plant Growth Assay

Plants treated with ANE at 1 g L^{-1} exhibited a significant increase in the number of nodules, over the control plants (Figure 5.1). The average number of nodules for control plants was 42, whereas it was 74 in plants treated with 1 g L^{-1} ANE. The average number of functional nodules of control plants was 39, whereas it was 44, 61, and 54 in plants treated with 0.5, 1, and 2 g L^{-1} ANE, respectively. However, no statistical difference in the number of functional nodules between the control and ANE-treated plants was observed (Figure 5.1).

Plants treated with 2 g L^{-1} ANE exhibited a significant increase in leaf area over the untreated plants (Figure 5.2); it was 87 cm^2 for 2 g L^{-1} ANE-treated plants while controls only had 71 cm^2 leaf areas. Other treatments did not significantly influence the leaf area as compared to the untreated control plants.

The average root length of control plants was 413 cm, whereas the values were 482, 500, 459, and 506 cm in plants treated with 0.1, 0.5, 1, and 2 g L^{-1} ANE, respectively. The results showed increased trends of total root length in ANE treated plants compared to untreated plants (Figure 5.3). However, there was no significant difference observed between untreated and ANE-treated plants for root length.

Similarly, there was no significant difference found between untreated control and ANE-treated alfalfa in root dry weights and shoot dry weights. The average number of root dry weight of control plants was 0.31 g, whereas it was 0.28, 0.32, 0.33 and 0.41 g in plants treated with 0.1, 0.5, 1, and 2 g L^{-1} ANE, respectively (Figure 5.4). The average

number of shoot dry weight of control plants was 0.51 g, whereas it was 0.50, 0.45, 0.60 and 0.56 g in plants treated with 0.1, 0.5, 1, and 2 g L^{-1} ANE, respectively (Figure 5.5).

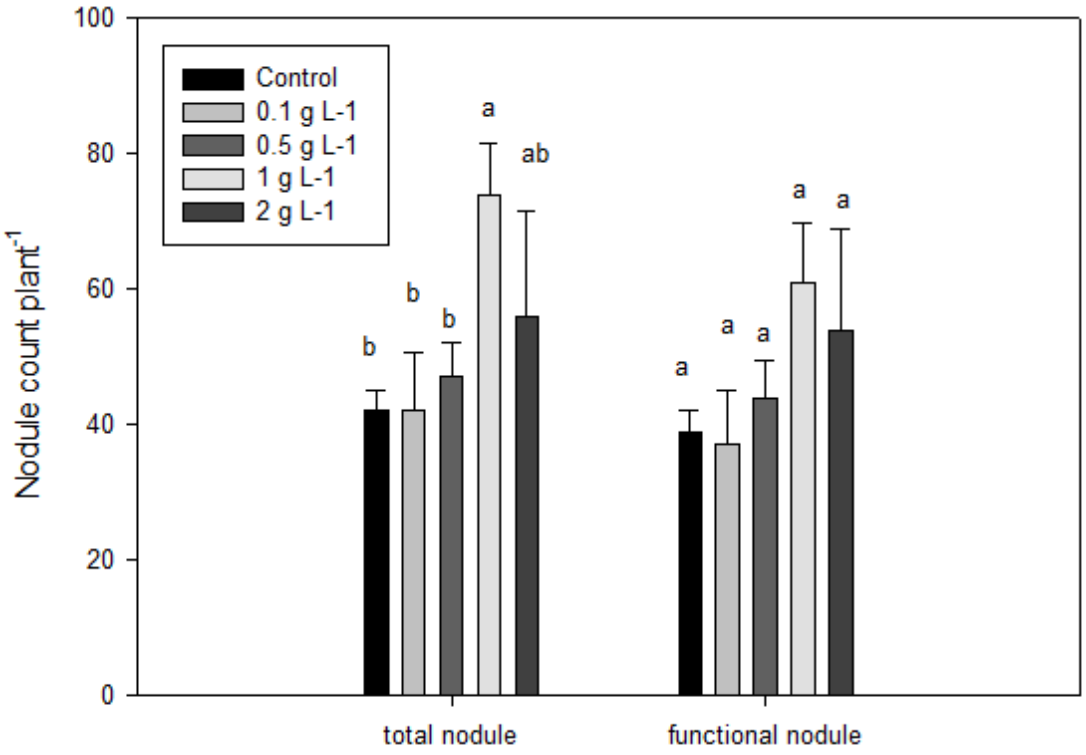


Figure 5.1. The effect of different concentrations of ANE on the number of nodules and number of functional nodules of alfalfa. Each value represents an average of 5 replicates. Bars indicate standard error.

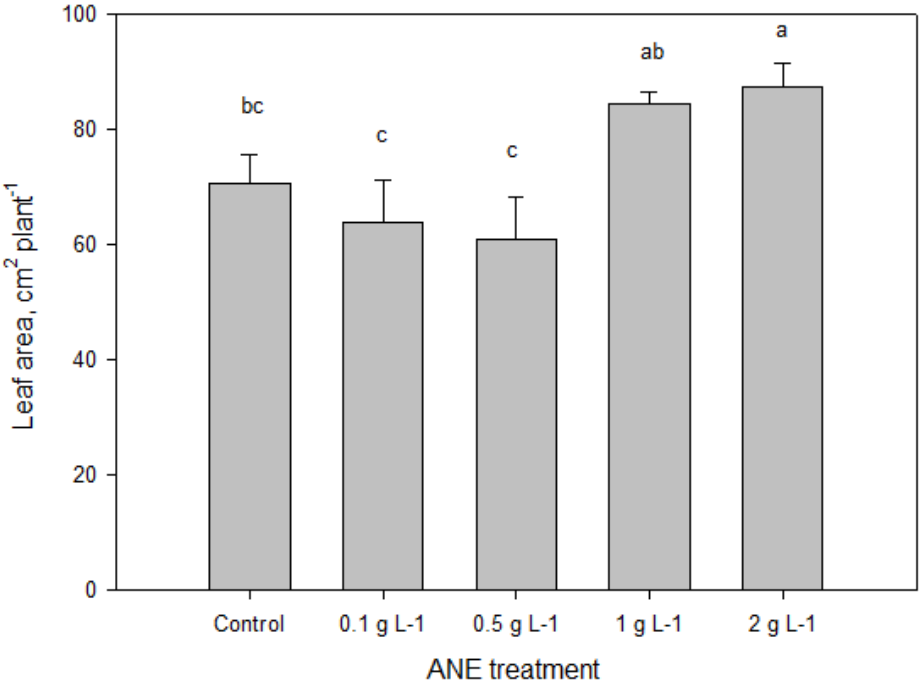


Figure 5.2. The effect of different concentrations of ANE on the leaf area of alfalfa. Each value represents an average of 5 replicates. Bars indicate standard error.

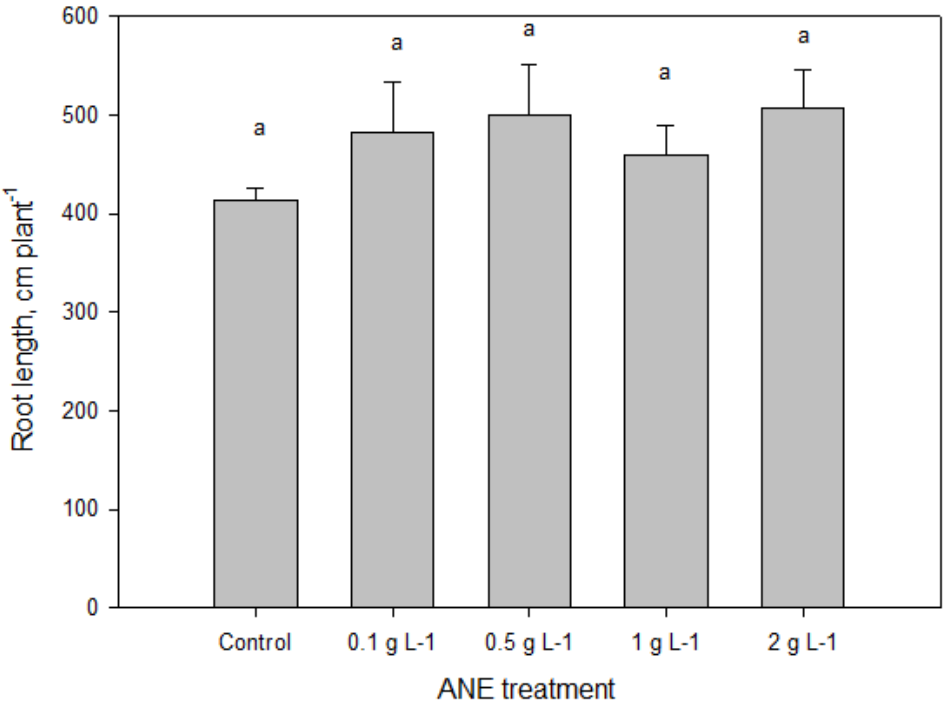


Figure 5.3. The effect of different concentrations of ANE on the root length in alfalfa. Each value represents an average of 5 replicates. Bars indicate standard error.

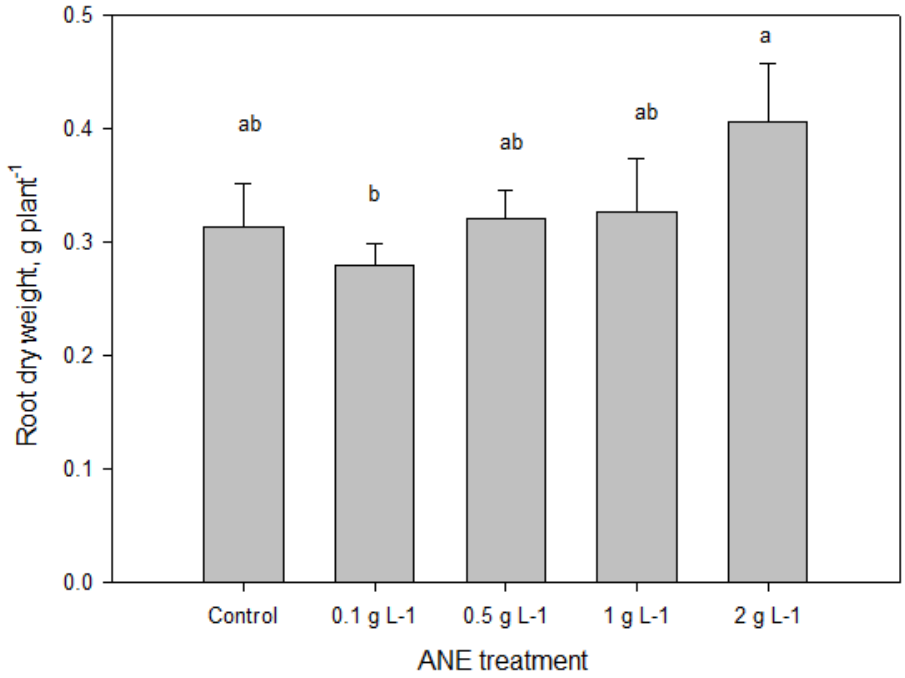


Figure 5.4. The effect of different concentrations of ANE on the root dry weight in alfalfa. Each value represents an average of 5 replicates. Bars indicate standard error.

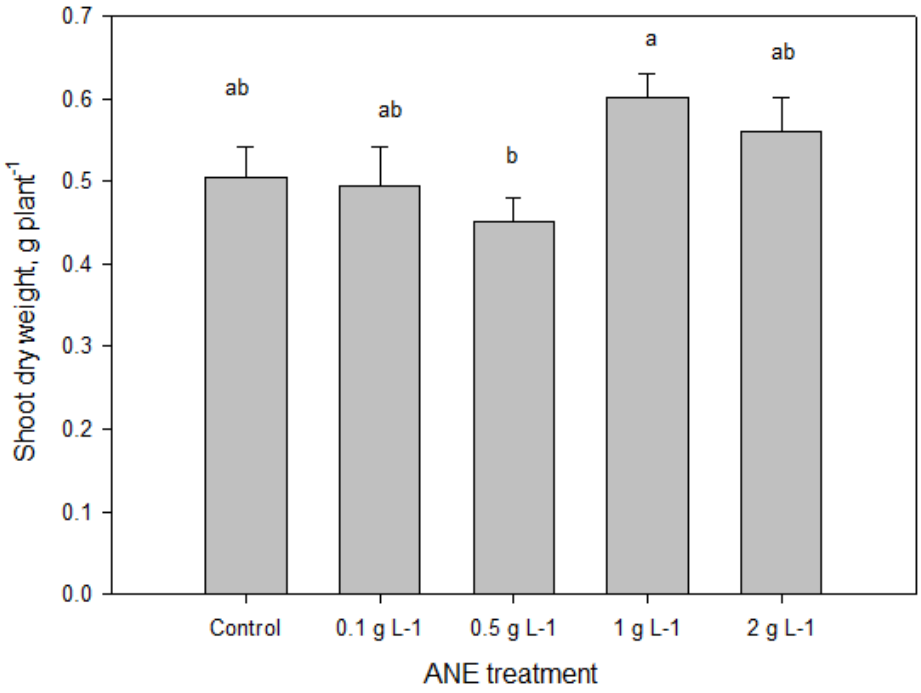


Figure 5.5. The effect of different concentrations of ANE on the shoot dry weight of alfalfa. Each value represents an average of 5 replicates. Bars indicate standard error.

5.1.2 Seed Priming Assay

Compared to untreated control plants, alfalfa treated with 0.5 g L^{-1} ANE had a significantly higher length of the whole plant (Figure 5.6). The total length of the control plants was 3.74 cm, whereas it was 4.34 cm in plants treated with 0.5 g L^{-1} ANE. Other treatments showed no significant influences on alfalfa length. The average values of alfalfa total length were 3.91 and 4.23 cm in ANE 0.25 and 1 g L^{-1} treated plants, respectively. However, the results showed increased trends of alfalfa total length in ANE treated plants compared to untreated controls.

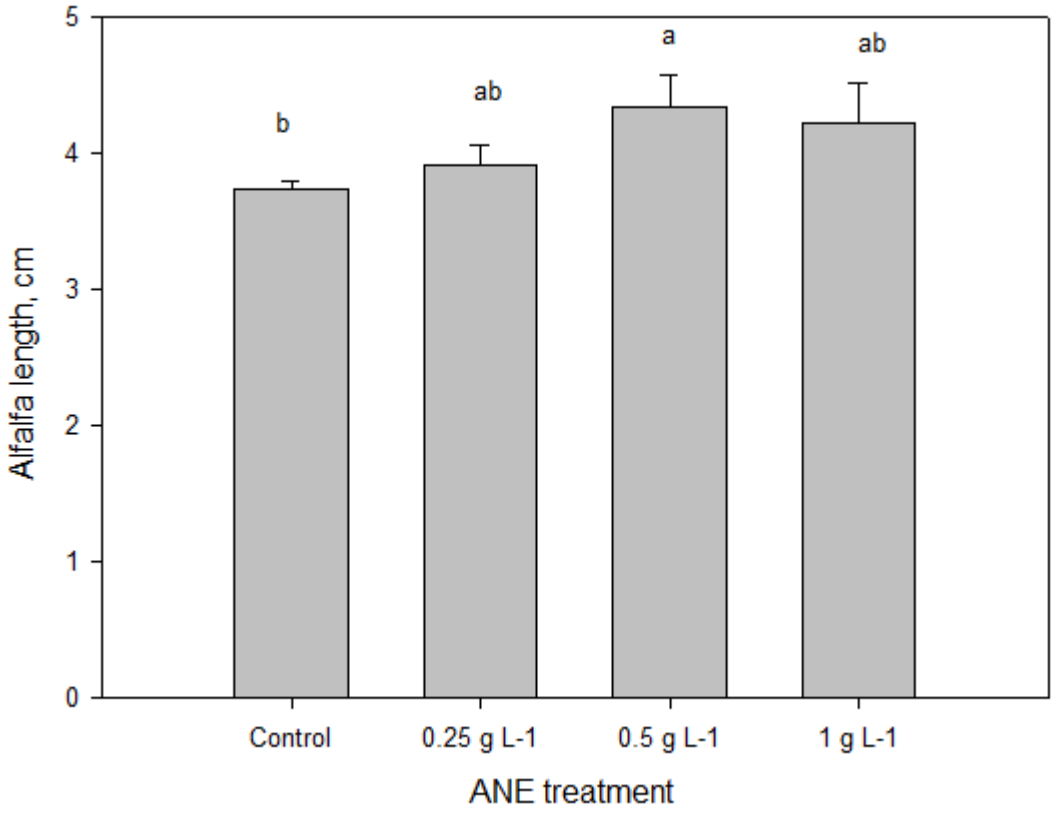


Figure 5.6. The effect of different concentrations of ANE on the alfalfa length. Each value represents an average of 15 replicates. Bars indicate standard error.

5.1.3 Seed Pelleting Assay

Compared to control plants, 75% ANE had a negative effect on total root length of alfalfa 5 days after planting. Alfalfa treated with 25% and 100% ANE exhibited significant increases in root length over the control plants 10 and 15 days after planting (Figure 5.7). The root length was 10.98 cm in control plants 10 days after planting while it was 14.25 and 15.42 cm in plants treated with 25% and 100% ANE. Other treatments had numerical increases in the mean value of root length as compared to controls 10 days after planting, though there were no statistical differences. The average root length was 17.44 cm in control plants 15 days after planting, while the root length in 25% and 100% ANE treated plants were 22.08 and 21.26 cm, respectively. Compared to control plants, 50% ANE had a negative effect on total leaf area of alfalfa 5 days after planting. However, plants treated with ANE all showed significant increases in leaf area as compared to controls 10 days after planting (Figure 5.8). The average leaf areas in 25%, 50%, 75%, and 100% ANE treated plants significantly increased and were 5.99, 4.78, 4.79, and 5.87 cm^2, respectively compared to the 3.48 cm^2 in the control. There was no significant difference observed between control and ANE-treated alfalfa 15 days after planting.

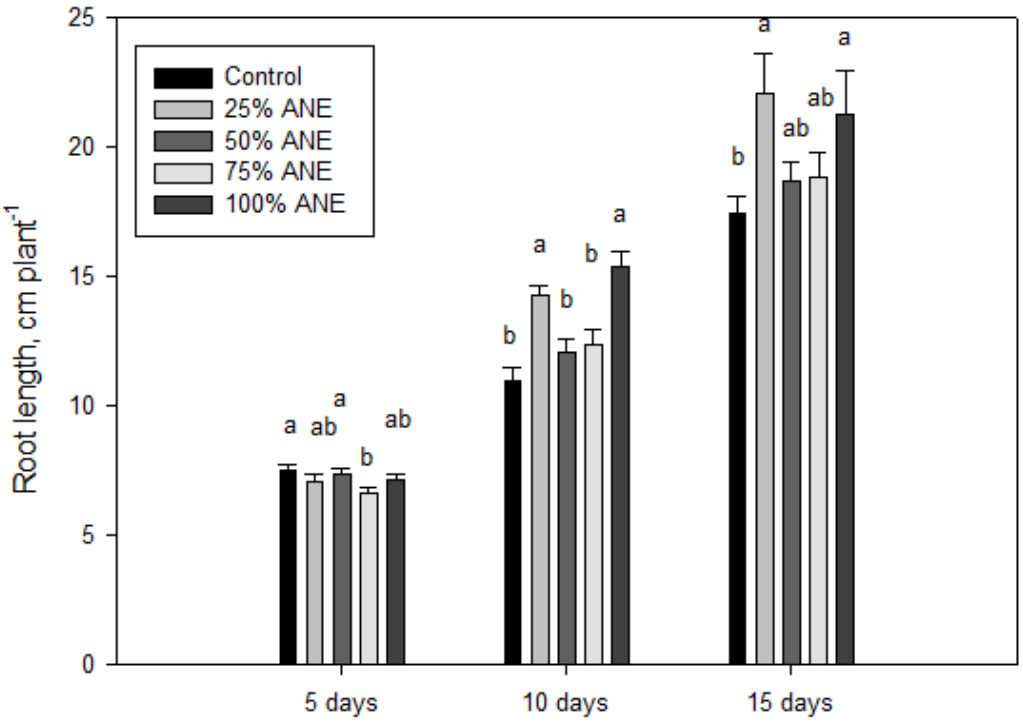


Figure 5.7. The effect of seed pelleting with different concentrations of ANE on root length of alfalfa. Each value represents an average of 15 replicates. Bars indicate standard error.

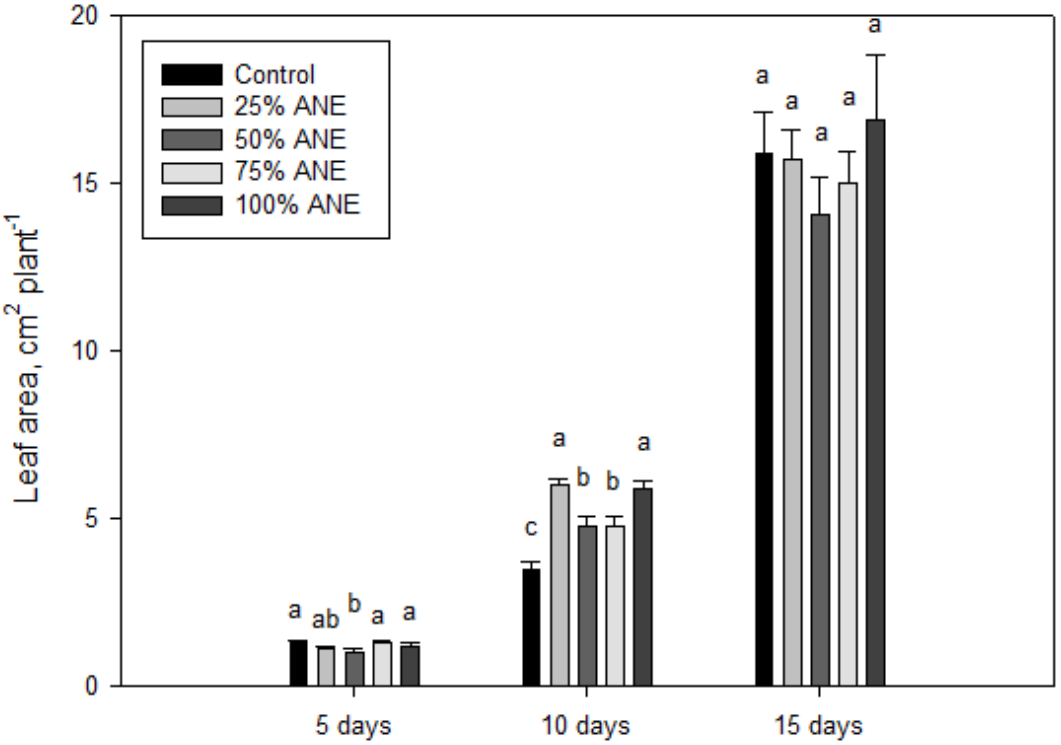


Figure 5.8. The effect of seed pelleting with different concentrations of ANE on leaf area of alfalfa. Each value represents an average of 15 replicates. Bars indicate standard error.

5.2 Laboratory Experiments

5.2.1 Root Hair Deformation Assay

ANE treatments had a significant influence on root hair deformation as compared to control plants 6 and 12 h post inoculation (Figure 5.9). Only 4% deformed root hairs was observed 6 h post inoculation in control plants, while 40% deformed root hairs was observed in ANE 0.5 g L^{-1} treated alfalfa. The percentage of the deformed root hairs in control plants increased to 24% 12 h post inoculation, while ANE 0.5 g L^{-1} treated alfalfa still had the most deformed root hairs with 42%. Though no significant increases were observed for controls and treated plants 24 h post inoculation, the results showed a similar trend as in 6 and 12 h post inoculation. Alfalfa treated with 0.5 g L^{-1} ANE exhibited a highest mean value of deformed root hairs with 50%. The number of deformed root hairs then decreased with the increased concentration of ANE.

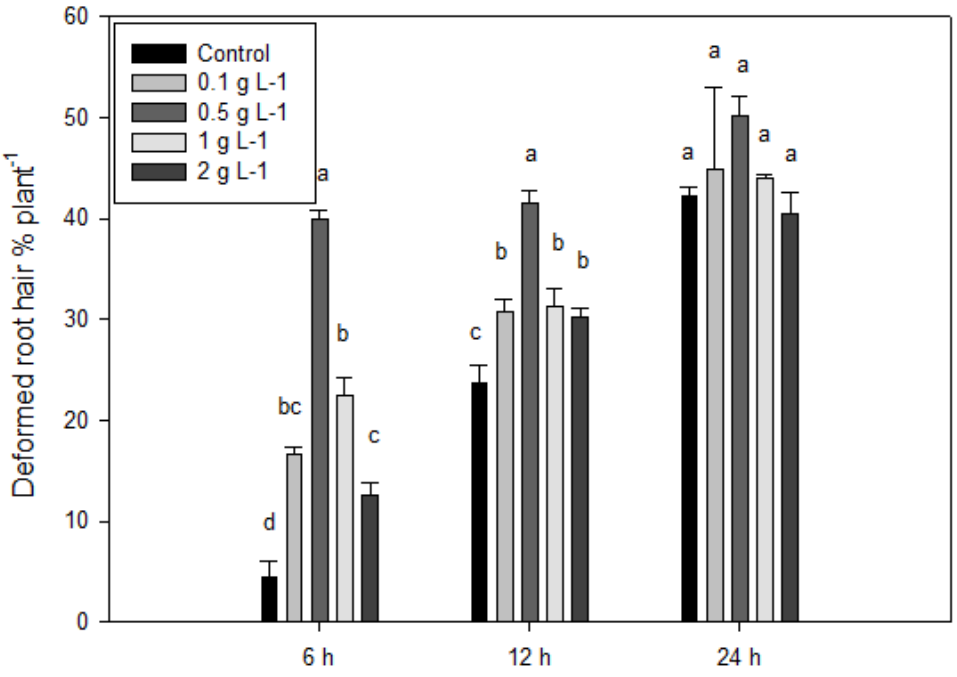
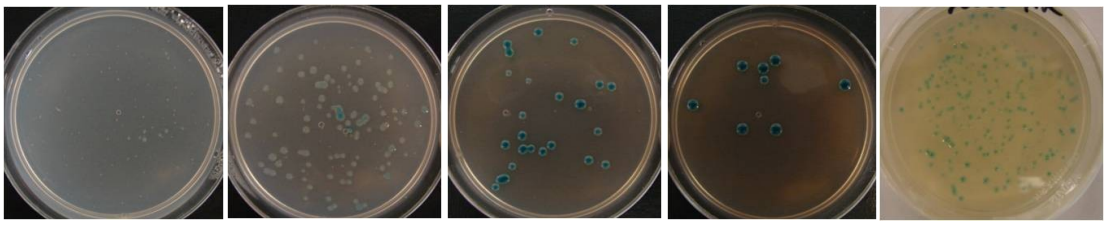


Figure 5.9. The effect of different concentrations of ANE on the root hair deformation of alfalfa. Each value represents an average of 3 replicates. Bars indicate standard error.

5.2.2 β-Galactosidase Assay

5.2.2.1 Qualification of the β-galactosidase Activity

Qualification of the β-galactosidase activity is shown in figure 5.10. Bacterial colonies were white on control plates, which indicated no or undetectable expression of *nodC* genes. Bacterial colonies turned blue on ANE-treated plates and luteolin (positive control) plates. With the increased concentration of ANE, the color of bacterial colonies turned darker, which suggested that higher concentration of ANE induced higher expression of *nodC* genes. However, as the concentration of ANE added to the minimal medium increased, the color of the medium also turned darker due to the color of the seaweed powder. The color of bacterial colonies could be the reflection of the background. Therefore, quantification of the β-galactosidase activity was conducted to confirm the results found from this experiment.



Control 0.1 g L^{-1} 0.3 g L^{-1} 0.5 g L^{-1} Luteolin

Figure 5.10. Qualification of the β-galactosidase activity of *S. meliloti* JM57 grown on minimal medium supplemented with different concentrations of ANE (0, 0.1, 0.3, and 0.5 g L^{-1}) and luteolin.

5.2.2.2 Quantification of the β-galactosidase Activity

The results of qualification of the β-galactosidase activity were confirmed by the quantification of the β-galactosidase activity. Although the number of Miller units was higher in the luteolin treatment than in ANE treatments, ANE treatment at 0.5 g L^{-1}

significantly increased, almost 2-fold the number of Miller units as compared to controls (Figure 5.11).

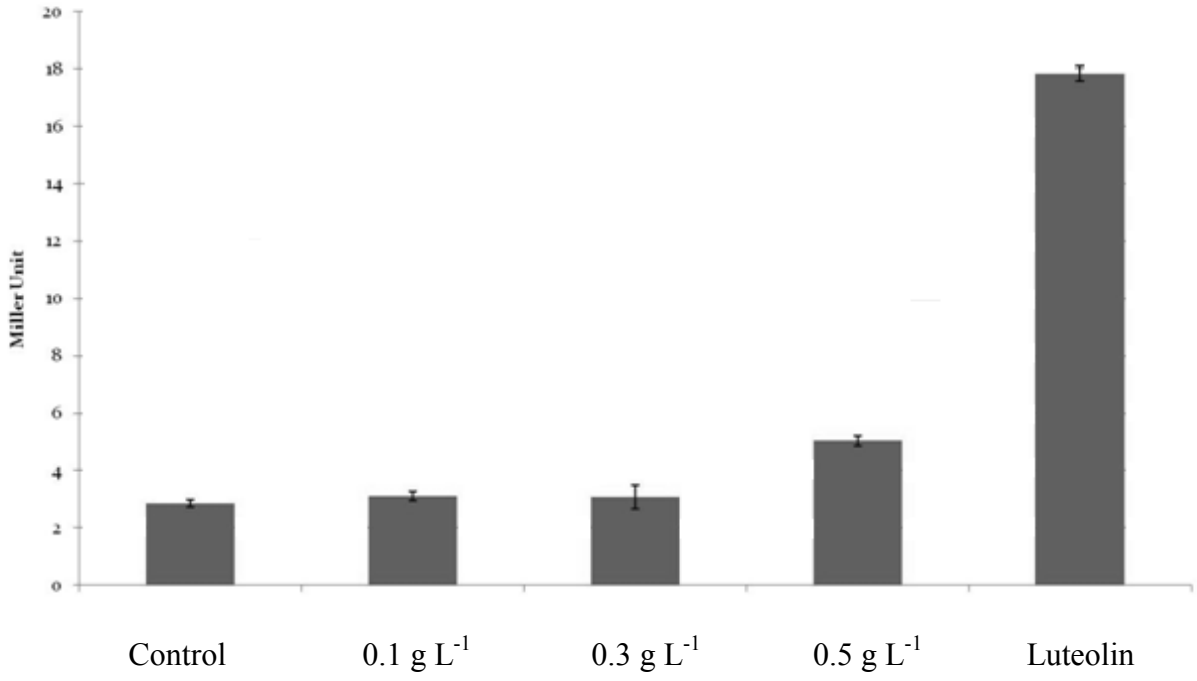


Figure 5.11. Quantification of the β-galactosidase activity of *S. meliloti* JM57 grown in liquid minimal medium supplemented with different concentrations of ANE (0, 0.1, 0.3, and 0.5 g L^{-1}) and luteolin. Each value represents an average of 3 replicates. Bars indicate standard error.

5.3 Field Experiments

5.3.1 Meteorological Conditions

The average daily temperatures measured for Nappan, NS during the study period did not frequently deviate from climate normal air temperatures (Figure 5.12a). The months of July and September in the year 2010 were slightly warmer than climate normals. Average monthly temperatures monitored during the growing seasons of 2009 and 2010 for Nappan were 15.4 and 15.2 °C (June), 17.5 and 19.9 °C (July), 18.6 and 18.4 °C (August), and 13.1 and 15.3 °C (September). The total precipitation received during the study period from June until September was 425 mm in the year 2009 and 422 mm in the

year 2010. In the year 2009, more than 60% of precipitation fell in July and August which was much more than average rainfall levels. In the year 2010, approximately 70% of precipitation fell in June and July. Rainfall was much less in August compared to climate averages (Figure 5.12b).

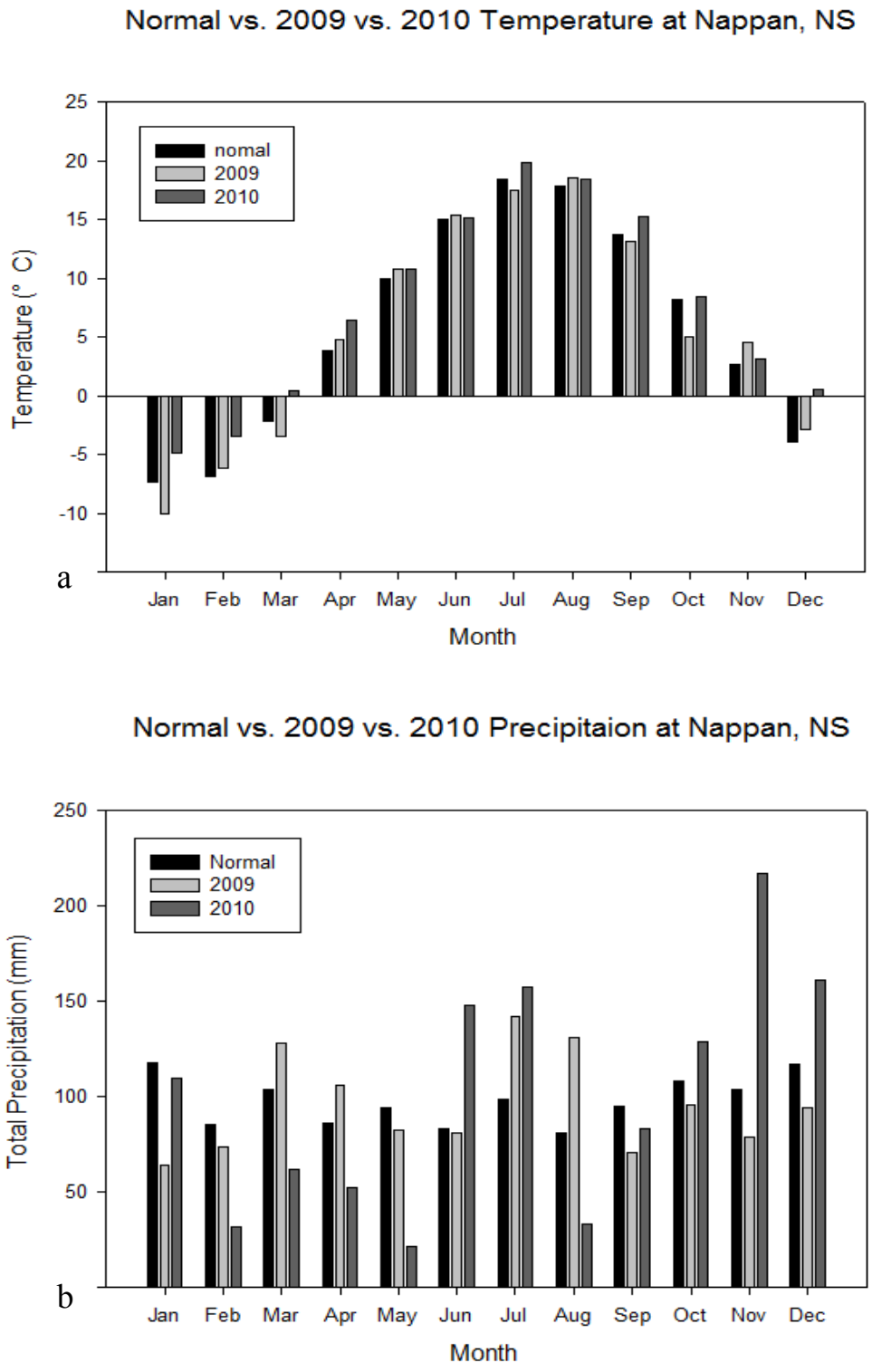


Figure 5.12. Weather conditions in Nappan, NS in 2009 and 2010 compared to average climate conditions 1971-2000: (a) normal vs. 2009 vs. 2010 temperature, and (b) normal vs. 2009 vs. 2010 precipitation (Data obtained from Environment Canada: http://www.climate.weatheroffice.gc.ca/).

5.3.2 Dry Matter Yield

The average dry matter yield in 2009 was 2.29 t ha^{-1}. The average dry matter yield in 2010 was 4.78 t ha^{-1}. In 2009, the plots treated with 1 g L^{-1} ANE had the highest yield at 2.43 t ha^{-1} while the highest yield in 2010 was 5.37 t ha^{-1} from the plots treated with 4 g L^{-1} ANE. Control and 0 g L^{-1} treatments were the least productive in years 2009 and 2010 (Figure 5.13). However, there were no significant differences observed between control and ANE-treated plant or among ANE treatments.

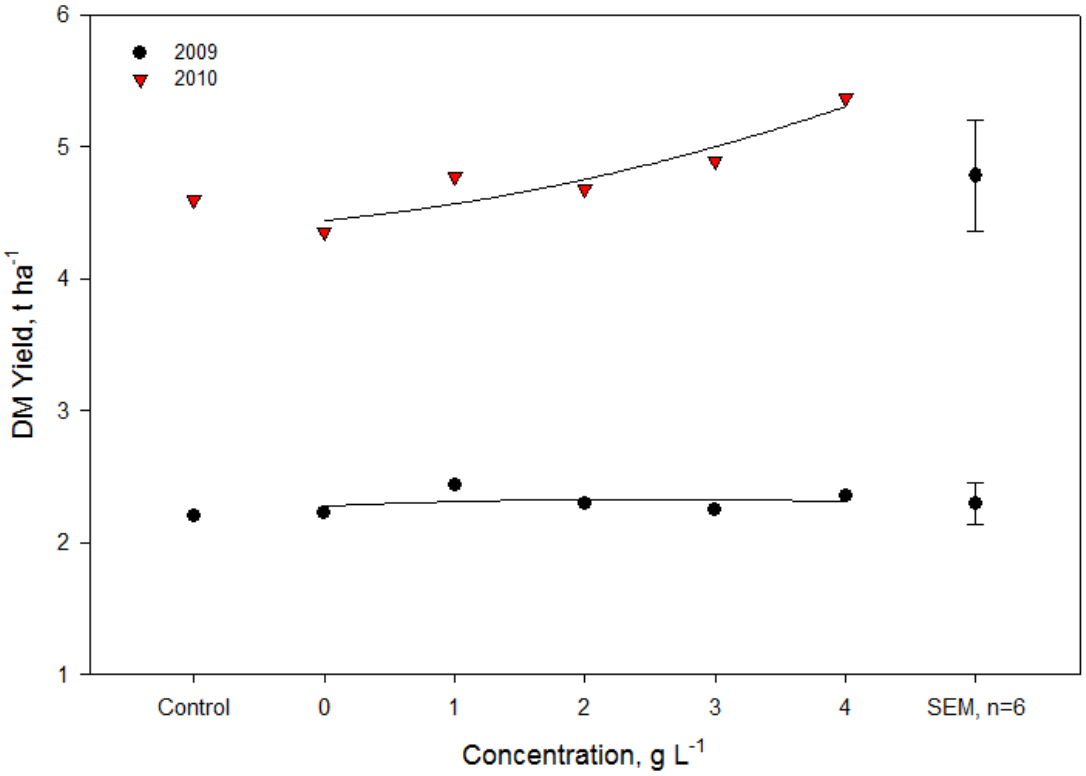
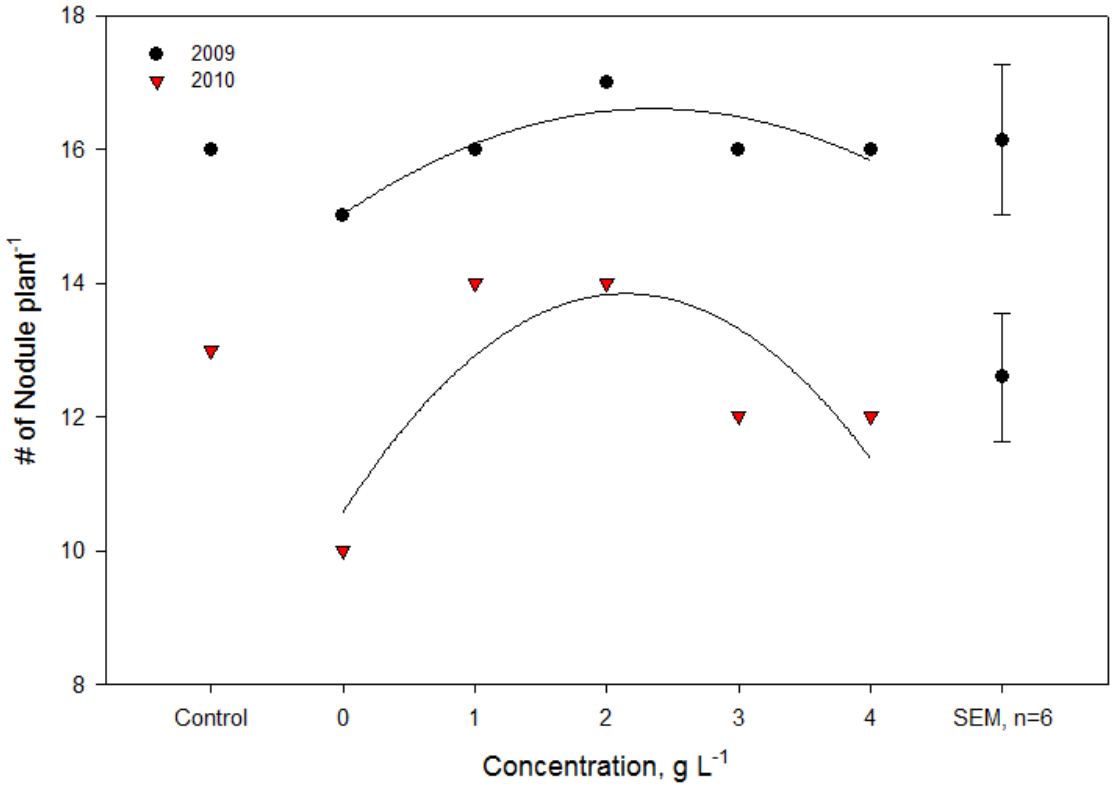


Figure 5.13. Mean annual dry matter yield during the establishment year (2009) and the post establishment year (2010) of alfalfa plots treated by varying rates of ANE.

5.3.3 Total Number of the Functional Nodules

Not many nodules were collected in either 2009 or 2010 due to the tough soil conditions when sampling by using a shovel. Only about 15 cm roots could be excavated. The average of the total number of functional nodules per plant in 2009 and 2010 was 16 and 13, respectively. There was no significant difference between control and treatments in both years 2009 and 2010 (Figure 5.14). No significant differences among each treatment were observed in the year 2009. However, in year 2010 there was a significant difference among treatments ($P = 0.01$) and ANE 1 g L^{-1} treatment produced the greatest number of the functional nodules while 0 g L^{-1} ANE treatment (water) was the least productive.

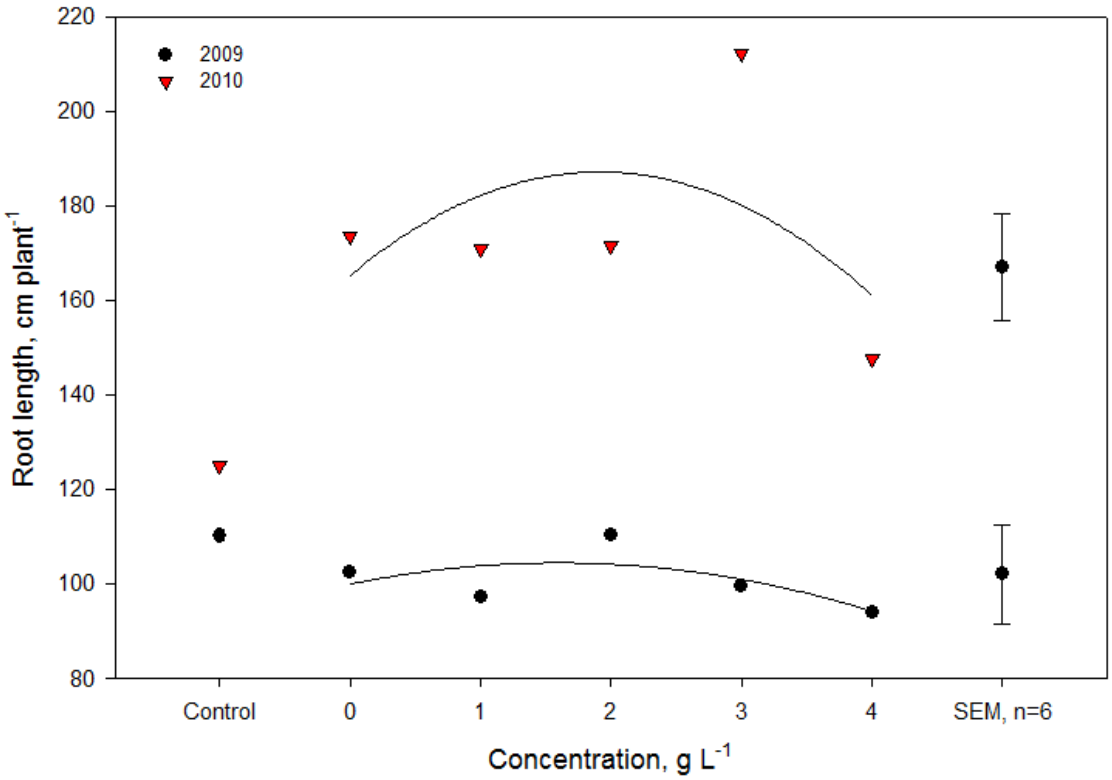


2009, (Ctrl vs Drench); p = NS
2010, (Ctrl vs Drench); p = NS
2009, Quad; p = NS
2010, Quad; p = 0.010

Figure 5.14. Mean total number of functional nodules per plant during the establishment year (2009) and the post establishment year (2010) of alfalfa plots treated by varying rates of ANE.

5.3.4 Total Root Length

The average total root length per plant in years 2009 and 2010 were102.19 cm and 166.75 cm, respectively. The statistical analysis showed no significant differences on average total root length among control or treatments in the year 2009 (Figure 5.15). On the contrary, the ANE treatments significantly ($P<0.001$) altered the root length compared to the control in the year 2010. The treatment ANE 3 g L^{-1} caused a statistically increase in average root length (212.20 cm) compared to the control (124.93 cm).

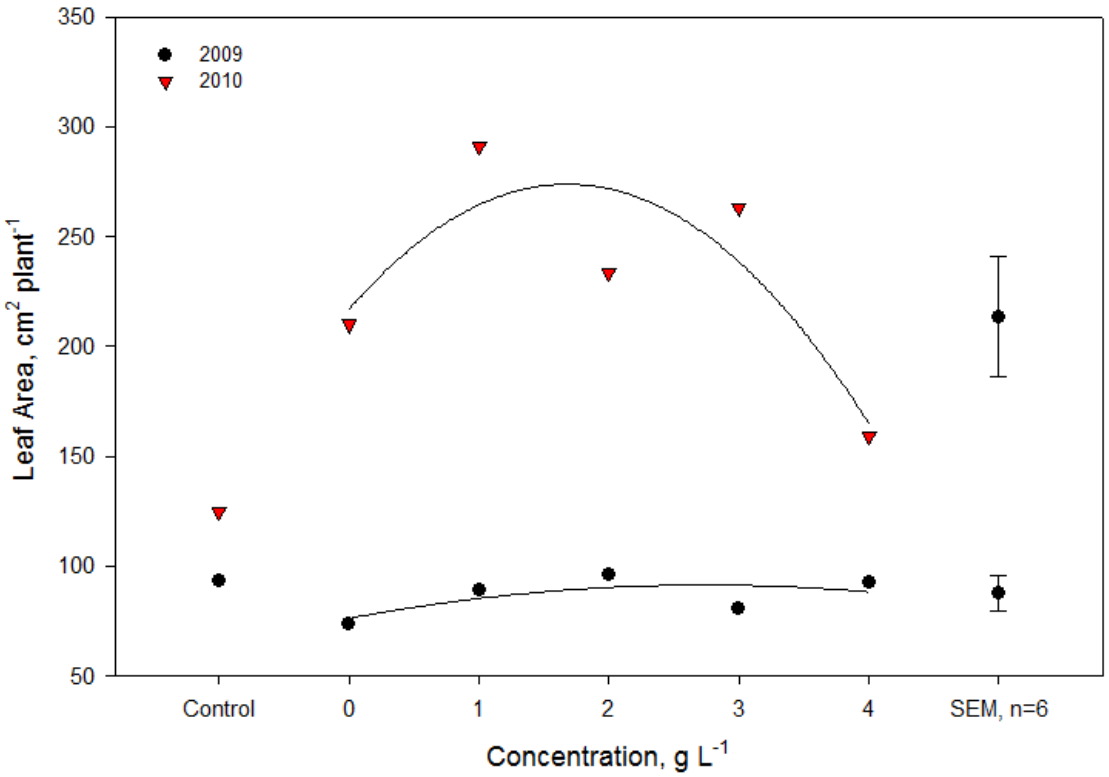


2009, (Ctrl vs Drench); p = NS
2010, (Ctrl vs Drench); p < 0.001

Figure 5.15. Mean total root length per plant during the establishment year (2009) and the post establishment year (2010) of alfalfa plots treated by varying rates of ANE.

5.3.5 Total Leaf Area

The average total leaf area per plant in years 2009 and 2010 were 87.34 cm^2 and 213.54 cm^2, respectively. There were not statistical differences between control and ANE-treated alfalfa or among ANE treatments in the year 2009 (Figure 5.16). In 2010, a significant difference between control and treatments (P = 0.002) was observed for the ANE 1 g L^{-1} treated plants where they showed an increase in the average leaf area (291.03 cm^2) compared to the control (124.90 cm^2).



2009, (Ctrl vs Drench); p = NS
2010, (Ctrl vs Drench); p = 0.002
2009, Quad; p = NS
2010, Quad; p = 0.011

Figure 5.16. Mean total leaf area per plant during the establishment year (2009) and the post establishment year (2010) of alfalfa plots treated by varying rates of ANE.

5.3.6 Root Dry Weight

The average root dry weight per plant in the years 2009 and 2010 were 0.32 and 1.81 g, respectively. Statistical analysis of average root dry weight showed no significant difference between control and other treatments or among treatments in either the years 2009 or 2010 (Figure 5.17).

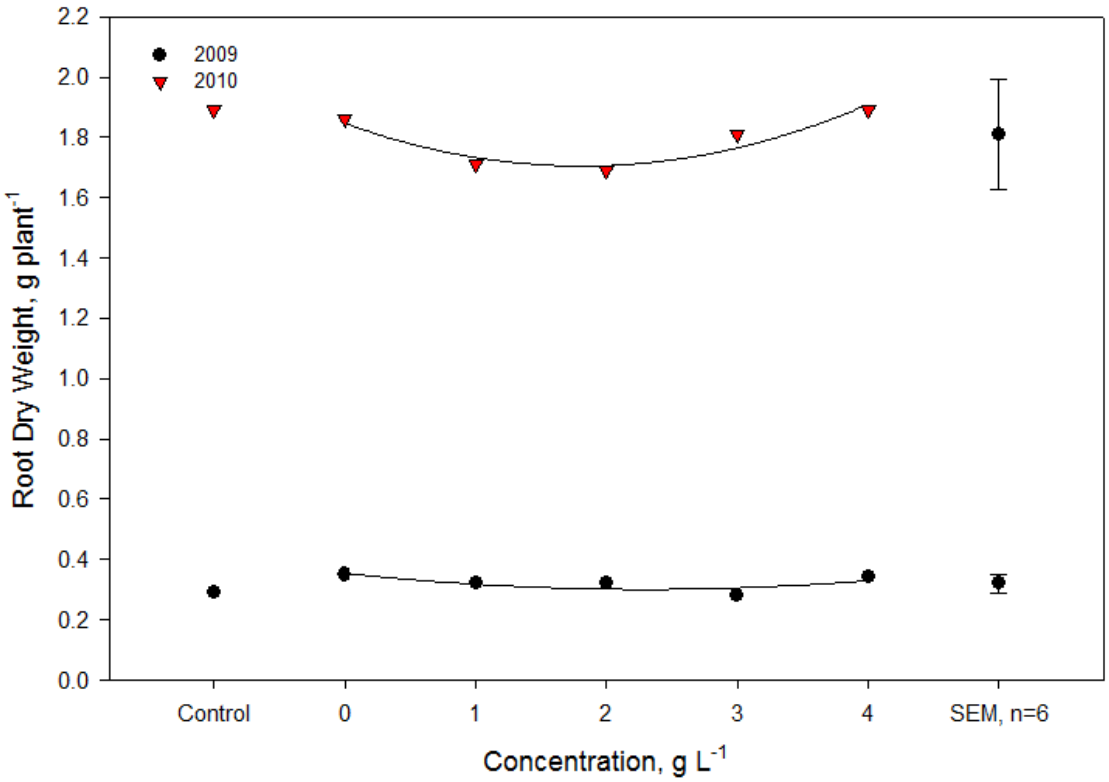


Figure 5.17. Mean root dry weight per plant during the establishment year (2009) and the post establishment year (2010) of alfalfa plots treated by varying rates of ANE.

5.3.7 Shoot Dry Weight

The average shoot dry weight per plant in the years 2009 and 2010 were 0.91 and 4.21 g, respectively. Similarly to root day weight, there were no significant differences between control and ANE-treated plant or among ANE treatments in either 2009 or 2010 (Figure 5.18).

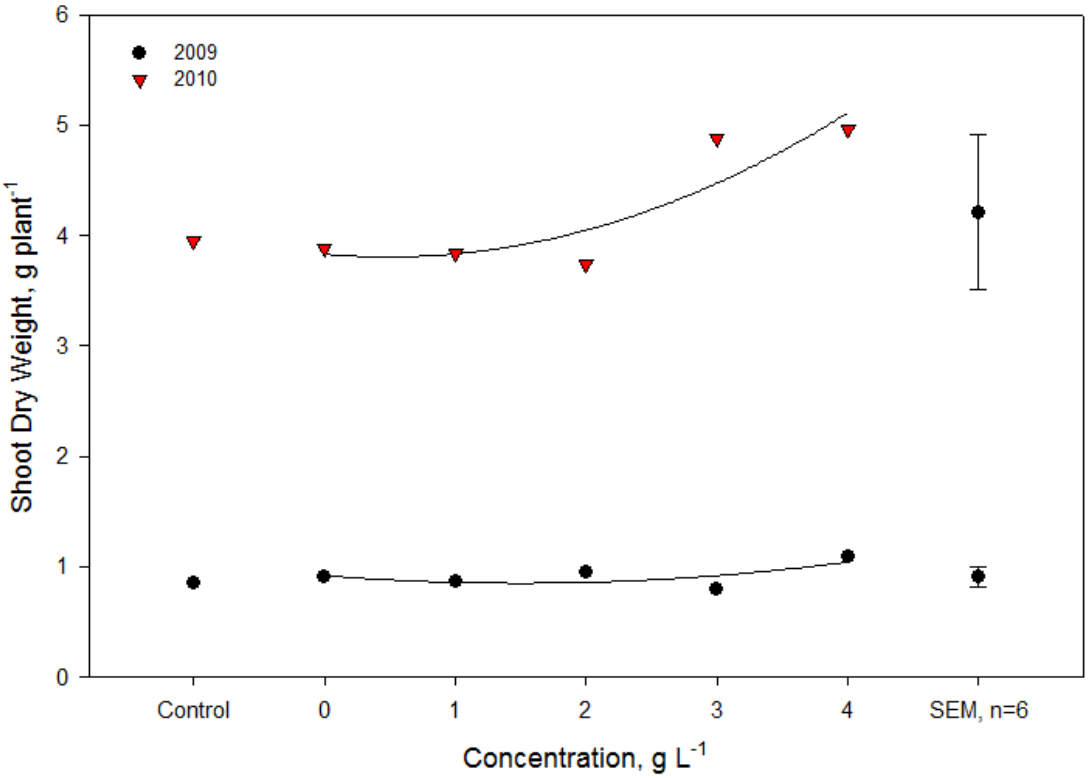


Figure 5.18. Mean shoot dry weight per plant during the establishment year (2009) and the post establishment year (2010) of alfalfa plots treated by varying rates of ANE.

5.3.8 Mineral Element Analysis

The macro and micro-element concentrations in ANE-treated and control plants are shown in Table 5.1. They were not significantly influenced by ANE treatments. However, alfalfa treated with 1 g L^{-1} ANE had more K, Fe, Mn, and Cu content compared to the control and other ANE treatments.

Table 5.1. Effects of different concentrations of ANE on some mineral elements (dry weight basis) in alfalfa leaves [a]

Trt	*N*	*P*	*K*	*Ca*	*Mg*	*Na*	*Fe*	*Mn*	*Cu*
	(mg/g)	**(mg/g)**	**(mg/g)**	**(mg/g)**	**(mg/g)**	**(mg/g)**	**(µg/g)**	**(µg/g)**	**(µg/g)**
Control	43.6a	3.6a	23.5a	22.6a	4.9a	0.6a	282.3a	80.6a	12.2a
0 g L-1	43.5a	3.7a	23.2a	22.0a	4.6a	0.7a	403.9a	80.3a	12.8a
1 g L-1	43.2a	3.5a	26.1a	22.0a	4.6a	0.6a	542.1a	91.1a	13.0a
2 g L-1	44.8a	3.6a	25.3a	23.2a	4.9a	0.5a	350.1a	84.9a	12.1a
3 g L-1	45.6a	3.6a	23.6a	22.0a	4.7a	0.6a	395.6a	85.2a	12.1a
4 g L-1	43.4a	3.5a	25.0a	22.3a	4.7a	0.6a	299.9a	78.6a	11.5a

[a] Values in the same column with the same letters are not significantly different ($P < 0.05$).

Chapter 6.0 Discussion

Given the currently increasing global population, there is a need for increased world food production. Plants play an important role as a food source for humans and a feed source for animals. In order to satisfy these demands, fertilizers, herbicides, pesticides, fungicides, and plant breeding have frequently been used to improve the quality and production of plants. However, there are serious concerns about the negative impacts of chemical products on human health and the ecosystem. From this perspective, it is important to explore the potential of naturally occurring plant-based products, such as biostimulants (e.g. substances extracts from marine algae), that can improve the growth and yield of plants with minimal adverse environmental impact.

A number of different algae have long been used to increase crop productivity when applied to soils as soil conditioning agents. The current commercial extracts are manufactured mainly from the brown seaweeds *Ascophyllum nodosum*, *Laminaria* spp., *Ecklonia maxima*, *Sargassum* spp., and *Durvillaea* spp. (Craigie 2010). Several studies have suggested that seaweed extracts have beneficial effects on plant growth, yield and biotic and abiotic stress tolerance due to the supply of essential nutrients, plant hormones and plant growth regulators contained in the seaweed extracts, and to improved soil texture and water holding capacity (Craigie 2010; Khan et al. 2012). Plants treated with seaweed extracts exhibit a wide range of responses, for instance, improved root development, growth and yield, leaf content of nutrients, resistance to biotic and abiotic stresses, have been well described in lots of studies (Temple and Bomke 1989; Verkleij 1992; Hong et al. 2007; Khan et al. 2009; Sunarpi et al. 2010).

Ascophyllum nodosum, one of approximately 2,000 species of brown seaweeds, is the most researched seaweed (Khan et al. 2009). Extracts of *A. nodosum* (ANE) have been used as fertilizers, biostimulants, and soil conditioners for a long time (Khan et al. 2012). Numerous studies have reported that ANE increase plant growth, yield and quality, fruit quality, improve the uptake of nutrient elements from soil, enhance phenolic antioxidant content in plants, impart plant resistance to pests and diseases, and mitigate the effects of environmental stresses such as salinity and freezing (Beryln and Russo1990; Verkleij 1992; Nabati et al. 1994; Rayorath et al. 2008b, 2009; Khan et al. 2009; Abdel-Mawgoud et al. 2010; Di et al. 2011). The mechanisms by which ANE affects plant metabolism are probably due to the carbohydrates, plant hormones, hormone-like plant growth regulators, trace elements, vitamins and amino acids contained in extracts (Challen and Hemingway 1965; Khan et al. 2009; Craigie 2010). These components affect cellular metabolism in plants associated with stress tolerance may be the cause of the increase plant growth. Temple and Bomke (1989) reported that foliar and soil application of ANE stimulated shoot growth and branching in bean crop. Khan et al. (2008) reported that ANE improved root nodulation in alfalfa. Rayorath et al. (2008a) also indicated that ANE promoted root and shoot growth in *Arabidopsis*. However, the physiological and molecular mechanisms responsible for the improvement in plants remain unclear.

Among 60 plant species in the genus *Medicago*, *M. sativa* (alfalfa) is the most widely cultivated perennial species. This crop predominates, among the legumes, in the supply of feed for livestock in Nova Scotia and other places. Alfalfa forage has about 17.5% crude protein and 2.8% nitrogen. Approximately 76.5% of the total nitrogen in the herbage is derived from symbiotic fixation (Peterson and Russele 1991). The present studies were

carried out to investigate the effect of ANE in growth and nodulation of alfalfa both in the greenhouse and under soil conditions. Moreover, the physiological and molecular basis of the growth improvement was studied.

The results of this thesis research demonstrated that application of ANE imparts effects on nodulation and growth of alfalfa, under both greenhouse and field conditions. Plant growth assay revealed that plants treated with ANE at 1 g L^{-1} exhibited a 1.8-fold increase in the number of nodule over the control plants. However, no significant difference was found on the number of functional nodules between control and ANE-treated plants. Alfalfa treated with 2 g L^{-1} ANE exhibited a significant increase in leaf area as compared to controls. No statistical differences were observed on the root length, root and shoot dry weight compared to control. Some concentrations of ANE (e. g. Figure 5.2) even had negative effects on alfalfa growth. The results here were different from a study conducted by Khan et al. (2008). They found that root irrigation with ANE significantly improved the total number of functional nodules and root dry weight of alfalfa. It is reported that under near optimal conditions, plants may have least response to seaweed products (Craigie 2010). Under certain conditions, such as adequate nutrient availability, seaweed extracts may even be inhibitory (Reitz and Trumble 1996). Reitz and Trumble (1996) also pointed out a particular ANE was not beneficial for stimulating the plant growth.

Soil salinization is one of the most important environmental factors affecting plant growth and yield all over the world. In legumes, high salinity suppresses photosynthesis and reduces the dry mass of shoots, roots, and nodules (Ibragimova et al. 2006). Since plants may have least response to seaweed products under ideal growth conditions, the

effect of ANE on the growth of alfalfa was evaluated in the greenhouse under the salt stress condition. In this study, alfalfa treated with 0.5 g L^{-1} ANE exhibited a significant increase in total length as compared to control plants. Nabati et al. (1994) also reported that in saline environment, seaweed extracts enhanced the growth and foliage weight, and stimulated rooting in Kentucky bluegrass. This improvement might be a result of an increase in the biosynthesis of endogenous cytokinins and betaine stimulated by ANE. Higher cytokinin concentrations in root systems help the transport of essential substances from roots to whole plants where they are needed (Nelson and Van Standen 1984). Betaines protect against drought, frost, high salinity or high temperature stresses in plants (Khan et al. 2009).

Seed pelleting with different coating materials is used to protect against unfavorable soil conditions. In legumes, seed pelleting with lime and gypsum increase nodulation, shoot and root growth in the saline alkali soil due to the protective influences (Chhonkar et al. 1971). Our seed pelleting assay showed ANE (75% and 50%) had a negative effect on root length and leaf area 5 days after planting. However, there was a significant increase in root length in ANE coated plants (25% and 100% ANE) 10 and 15 days after planting. It was interesting to find a similar pattern in root length 10 and 15 days after planting that lower concentration of ANE (25%) and higher concentration of ANE (100%) had significant effects on plants, whereas 50% and 75% ANE did not influence on alfalfa. The reason was unclear. ANE treatments also improved the leaf area of alfalfa 10 days after planting. However, there was no significant difference in leaf area between control and ANE-treated plants 15 days after planting. The significant increase in root length of ANE-treated plants is probably, at least in part, attributed to the increase of endogenous

plant hormones stimulated by ANE when seeds start to germinate (Reitz and Trumble 1996). This suggests that ANE treatment through seed pelleting may induce specific systemic physiological responses leading to promotion of root length and leaf area.

The ANE treatments were applied as root irrigation or seed treatment, but resulted in an enhancement of growth at the whole plant level. These physiological, biochemical and molecular changes in extract treated plants were investigated using a combination of biochemical and molecular analyses.

Root hair deformation is frequently used to study the activity of Nod factors since it is a simple and rapid assay. Nod factors trigger the root hair deformation at early stage of nodulation. It is reported that 5 to 10 min of Nod factor-root interaction is sufficient to induce root hair deformation in vetch (Heidstra et al. 1994). In this study, alfalfa treated with ANE at 0.5 g L^{-1} had approximately 40% deformed root hairs 6 h post inoculation, whereas control plants only had 4% deformed root hairs. Other treatments also caused 3 to 6-fold increases in deformed root hairs. The results of 6, 12, and 24 h post inoculation were following a similar trend that most deformed root hairs were observed in ANE 0.5 g L^{-1} treated alfalfa. The number then decreased with the increased concentration of ANE (Figure 5.9). It is reported that when alfalfa plant roots were treated with ANE before *Sinorhizobium meliloti* inoculation, the number of bacteria present or attached to the root hairs increased (Khan et al. 2012). This could be due to the stimulation by ANE treatment of the alfalfa roots to produce specific secondary metabolites, flavonoids for instance, thus attracting *S. meliloti* to the root hairs and accelerating root hair deformation. The increase in the number of deformed root hairs also suggested that ANE may contain compounds, which are able to induce *nod* genes expression and therefore stimulate the

synthesis of Nod factors secreted by *S.meliloti*. In order to confirm this hypothesis, a β-galactosidase assay was conducted to investigate the molecular basis of ANE effects on nodulation in alfalfa, specifically on the expression of *nodC* genes of the associated rhizobia. The rhizobium-legume symbiosis begins with a signal molecule exchange process between the two potential partners. Flavonoids secreted by the roots of legumes trigger the expression of the *nod* genes, resulting in the synthesis of Nod factors. Both qualitative and quantitative analysis of the β-galactosidase activity revealed that ANE treatments at 0.5 g L^{-1} significantly increased the expression of *nodC* genes, which encode the proteins essential to make the core Nod factors structure, as compared to controls. The results here were consistent with the results from root hair deformation assay. β-galactosidase results suggested that ANE may act in a way similar to flavonoids in the rhizobium-legume symbiosis. However, further studies are needed to determine the nature of the chemical(s) present in the ANE.

The average daily temperatures measured for Nappan, NS during the field study period did not frequently deviate from climate normal air temperature. The total precipitation received during the study period at the Nappan site was almost the same in years 2009 and 2010, although slightly more than climate averages. In the establishment year (2009), statistical analysis showed that ANE treatments did not significantly modify the nodulation and growth of alfalfa. However, in the post-establishment year (2010), results showed that ANE treatments increased the total number of functional nodules, total root length and total leaf area. No significant differences were observed in the total dry matter yield, root and shoot dry weight. In 2010, alfalfa treated with ANE 3 g L^{-1} exhibited a significant increase in total root length (Figure 5.15), whereas ANE did not

influence on root dry weight (Figure 5.17). The discrepancy could be due to the different sampling sizes. The total root length was measured based on 5 plants per plot, while the roots were weighed based on 10 plants per plot. Similarly, alfalfa treated with ANE 1 g L^{-1} exhibited a significant increase in total leaf area (Figure 5.16), the results were based on 5 plants per plot, whereas ANE did not influence on shoot dry weight (Figure 5.18) based on 10 plants per plot. The statistical analyses were summarized in Appendix.

Rathore et al. (2009) found that the application of *Kappaphycus alvarezii* extract, a species of red alga, enhanced the growth parameters measured for soybean, e.g. plant height, number of plants per square meter, number of branches per plant and yield. They also reported that with increasing concentration of seaweed extract, those growth parameters gradually increased. Similar results were also found in rice (Sunarpi et al. 2010) and watermelon (Abdel-Mawgoud et al. 2010). As mentioned before, under different conditions, seaweed products are not beneficial on plants, or may even be inhibitory. The different degree of response caused by seaweed extract application could be due to application rates, frequency and timing of the treatments in different season, geographical location and local environmental conditions (Craigie 2010). Under greenhouse conditions, high concentrations of ANE (> 5 g L^{-1}) have negative impacts on plants, probably because they contain large amounts of sodium and potassium. Under field conditions, there are too many variables that cannot be controlled, such as climate factors, soil conditions and biotic factors, therefore lower concentrations of ANE may not have significant effects on plant growth and development.

In the field study, ANE at 1 g L^{-1} significantly increased the total number of functional nodules and total leaf area on alfalfa in 2010. ANE at 3 g L^{-1} significantly

improved the total root length. Earlier experiment in our laboratory showed that ANE at 1 g L^{-1} significantly improved the total number of functional nodules (up to 100/plant), and root dry weights of alfalfa in the greenhouse (Khan et al. 2008; Khan et al. 2012). In the present work, no significant effect of ANE was found in regard to root dry weights between the control and other treatments. Although ANE treatment showed a significant increase in the total number of functional nodules, the numbers excavated per plant were very low (average 14/plant). There were numerous nodules remained in the soil. The difference between previous findings and our results here was due to the specific root growth habits of alfalfa in the field and the soil conditions. Alfalfa is able to form a deep and extensive root system, penetrating to 3 to 5 m into the soil in order to bring up water and nutrients. The taproots near the soil surface could reach 5 to 10 mm in diameter. About 30 to 50 cm under the soil surface, abundant lateral roots usually grow parallel with the soil surface up to 30 cm and therefore the soil could be all occupied by the roots of alfalfa. Nodules could be present throughout the whole root system but abundant nodules are found in the surface 50 to 60 cm of soil (Burton 1972). Meanwhile, the soil was dry and tough with lots of small rocks when samples were excavated. Only about 15 cm roots could be excavated. Taken all together, this root habits in the field and the soil texture increased the difficulties of simple sampling with a shovel.

Previous studies have found that the application of seaweed extract significantly increases the macro and micro-element concentrations in plants. Rathore et al. (2009) reported that using seaweed extract from *Kappaphycus alvarezii* increased N, P, and K uptake by soybean. Butler and Hunter (2006) found the application of ANE increased leaf tissue N, P, K, and Fe in turfgrass. Chouliaras et al. (2009) reported that ANE increased

K, Fe, and Cu in olive leaves. Previous studies in our laboratory also showed that ANE application at 1 g L^{-1} significantly increased K and Fe concentrations in spinach leaves (unpublished data). However, our results showed no significant increase of the macro- and micro-element concentrations in ANE treated alfalfa leaves.

The application of ANE influences growth parameters, for instance, number of functional nodules, root length, and leaf area of alfalfa. The difficulties of the field study came from the soil conditions, weather conditions, plant habits, the determination of the concentration and amount of ANE used in the study, and the methods used for sampling and plant cleaning.

In conclusion, we found that ANE-modulated specific metabolic pathways both in alfalfa and the bacterium, resulting in enhanced *rhizobium*-legume symbiosis. The effect of ANE on alfalfa is probably due to its numerous components such as carbohydrates, plant hormones, hormone-like plant growth regulators, trace elements, vitamins and amino acids. This naturally occurring plant-based product can improve the growth and yield of plants but have less adverse environmental impact thus the investigations into the mechanism(s) by which ANE treatment may exert such beneficial effects on alfalfa have to be further extended. Although ANE treatment has no direct effect on the growth number of bacteria (Khan et al. 2012), it does have an influence on the induction of *nodC* gene expression. Analysis of the effect of ANE on Nod factors and other *nod* genes, such as *nodD*, as well as early nodulin genes induced in alfalfa (e.g. Enod11 and Enod12 gene) should be further studied.

References

Abdel-mawgoud, A. M. R., Tantaway, A. S., Hafez, M. M., and Habib, H. A. M. 2010. Seaweed extract improves growth, yield and quality of different watermelon hybrids. Res. J. Agric. & Biol. Sci. **6:** 161-168.

Aitken, J. B., and Senn, T. L. 1965. Seaweed products as a fertilizer and soil conditioner for horticultural crops. Bot. Mar. **8:** 144-148.

Alam, M. Z., Braun, G., Hodges, D. M., and Norrie, J. 2010. The effects of a commercial extract of the brown seaweed *Ascophyllum nodosum* on plant growth and soil microbial dynamics in strawberry. Proceedings of the 37th Annual Meeting of the Plant Growth Regulation Society of America, Portland, Oregon, USA, 8-12 August 2010.

Al-Falih, A. M. K. 2002. Factors affecting the efficiency of symbiotic nitrogen fixation by Rhizobium. Pak. J. Biol. Sci. **5**: 1277-1293.

Asadi, A., Khavazi, K., Afshari, M., and Ghasemi, M. 2011. Increasing the efficiency of biological nitrogen fixation, increasing the function and decreasing nitrogen fertilizers consumption in soya under cultivation farms. Adv. Environ. Biol. **5:** 2365-2368.

Badri, D. V., Weir, T. L, van der Lelie, D., and Vivanco, J. M. 2009. Rhizosphere chemical dialogues: plant-microbe interactions. Curr. Opin. Biotechnol. **20**: 642-650.

Bailly, X., Béna, G., Leniefa, V., de Lajudiea, P., and Avarre, J. C. 2006. Development of a lab-made microarray for analyzing the genetic diversity of nitrogen fixing symbionts *Sinorhizobium meliloti* and *Sinorhizobium medicae*. J. Microbiol. Met. **67**: 114-124.

Barran, L. R., Bromfield, E. S. P., and Brown, D. C. W. 2002. Identification and cloning of the bacterial nodulation specificity gene in the *Sinorhizobium meliloti – Medicago laciniata* symbiosis. Can. J. Microbiol. **48**: 765-771.

Beryln, G. P., and Russo, R. O. 1990. The use of organic biostimulants in nitrogen fixing trees. Nitrogen Fixing Tree Res. Rep. **81:** 1-2.

Barnett, M. J. and Fisher, R. F. 2006. Global gene expression in the rhizobial–legume symbiosis. Symbiosis **42:** 1–24.

Bertin, C., Yang, X., and Weston, L. A. 2003. The role of root exudates and allelochemicals in the rhizosphere. Plant Soil **256**: 67-83.

Blunden, G., Cripps, A. L., Gordon, S. M., Mason, T. G., and Turner, C. H. 1986. The characterization and quantitative estimation of betaines in commercial seaweed extracts. Bot. Mar. **29**: 155-160.

Bouwmeester, H. J., Roux, C., Lopez-Raez, J. A., and Becard, G. 2007. Rhizosphere communication of plants, parasitic plants and AM fungi. Trends Plant Sci. **12**: 224-230.

Brady, N. C., and Weil, R. R. 2002. The nature and properties of soils. Prentice Hall, Upper Saddle River, N.J.

Brewin, N. J. 2004. Plant cell wall remodelling in the *Rhizobium*–Legume symbiosis. Crit. Rev. Plant Sci. **23**: 293–316.

Brockwell, J., Pilka, A., and Holliday, R. A. 1991. Soil pH is the major determinant of the numbers of naturally-occurring *Rhizobium meliloti* in non-cultivated soils in New South Wales. Aust. J. Agr. **31:** 211-219.

Burton, J. C. 1972. Nodulation and symbiotic nitrogen fixation. In: Alfalfa Science and Technology. (Hanson, C. H. Ed.). American Society of Agronomy, Inc., Madison, Wisconsin, USA, Chapter 11.

Bustamante, M. M. C., Medina, E., Asner, G. P., Nardoto, G. B., and Garcia-Montiel, D. C. 2006. Nitrogen cycling in tropical and temperate savannas. Biogeochemistry **79:** 209-237.

Butler, T, and Hunter, A. 2006. Impact of seaweed extract on turfgrass growth and nutrition on a golf green to USGA Specification. Acta Hort (ISHS) **762**: 81-90.

Capela, D., Barloy-Hubler, F., Gouzy, J., Bothe, G., Ampe, F., Batut, J., Boistard, P., Becker, A., Boutry, M., Cadieu, E., Dreano, S., Gloux, S., Godrie, T., Goffeau, A., Kahn, D., Kiss, E., Lelaure, V., Masuy, D., Pohl, T., Portetelle, D., Puhler, A., Purnelle, B., Ramsperger, U., Renard, C., Thebault, P., Vandenbol, M., Weidner, S., and Galibert, F. 2001. Analysis of the chromosome sequence of the legume symbiont Sinorhizobium meliloti strain 1021. PNAS **98:** 9877-9882.

Cardozo, K., Guaratini, T., Barros, M. P., Falcão, V. R., Tonon, A. P., Lopes, N. P., Campos, S., Torres, M. A., Souza, A. O., Colepicolo, P., and Pinto, E. 2007. Metabolites from algae with economical impact. Comparative Biochem. Physiol. Part C **146:** 60-78.

Challen, S. B., and Hemingway, J. C. 1965. Growth of higher plants in response to feeding with seaweed extracts. Proc. 5th Ind. Seaweed Symp.

Chhonkar, P. K., Iswaran, V., and Jauhri, K. S. 1971. Seed pelleting in relation to nodulation and nitrogen fixation by *Phaseolusaureus* L. in a saline alkali soil. Plant and Soil **35:** 449-452.

Chouliaras, V, Tasioula, M., Chatzissavvidis, C., Therios, I., and Tsabolatidou, E. 2009. The effects of a seaweed extract in addition to nitrogen and boron fertilization on productivity, fruit maturation, leaf nutritional status and oil quality of the olive (*Olea europaea* L.) cultivar Koroneiki. J. Sci. Food Agric. **89**: 984-988.

Cooper, J. E. 2004. Multiple responses of rhizobia to flavonoids during legume root infection. Adv. Bot. Res. **41:** 1–62.

Cooper, J. E. 2007. Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. J. Appl. Microbiol. **103**: 1355-1365.

Cooper, J. B., and Long, S. R. 1994. Morphogenetic rescue of *Rhizobium meliloti* nodulation mutants by trans-zeatin secretion. Plant Cell **6**: 215-225.

Craigie, J. S. 2010. Seaweed extract stimuli in plant science and agriculture. J. Appl. Phycol. **23:** 371-393.

Crews, T. E., and Peoples, M. B. 2004. Legume versus fertilizer sources of nitrogen: Ecological tradeoffs and human needs. Agriculture, Ecosystems & Environment **102:** 279.

Crouch, I. J., Beckett, R. P., and Van Staden, J. 1990. Effect of seaweed concentrate on the growth and mineral nutrition of nutrient stressed lettuce. J. Appl. Phycol. **2:** 269-272.

Delwiche, C. C. 1970. The nitrogen cycle. Scientific American **223:** 137-146.

Dénarié, J., Debellé, F., and Promé, J. C. 1996. Rhizobium lipo-chitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. Annual Rev. Biochem. **65**: 503-535.

Douglas, B. 1998. Nitrogen sources in potato fertilizers. Soil and Feed Testing Laboratory. http://www.gov.pe.ca/agriculture/index.php3?number=71711&lang=E-.

Druart, N., Goupil, P., Dewaele, E., Boutin, J., and Rambour, S. 2000. Nitrate assimilation in chicory roots (*Cichorium intybus* L.) which acquire radial growth. J. Exp. Bot. **51:** 539-546.

Eaglesham, A. R. J., and Ayanba, A. 1984. Tropical stress ecology of rhizobia, root nodulation and legume fixation. In: Current Developments in Biological Nitrogen Fixation. (Subba Rao, N. S. Ed.). Edward Arnold. Maryland, USA, pp: 1-35.

Eivazi, A. R., Fajri, A., Rezazad, M., Soleimapour, M., and Rezai, M. 2012. Evaluation of potential of biological nitrogen fixation of Rhizobium strains in legume crops in West Azerbaijan province. Iran. J. Crop Sci. **13:** 627-641.

Fan, D., Hodges, D. M., Zhang, J., Kirby, C. W., Ji, X., Locke, S. J., Critchley, A. T., and Prithiviraj, B. 2011. Commercial extract of the brown seaweed *Ascophyllum nodosum* enhances phenolic antioxidant content of spinach (*Spinacia oleracea* L.) which protects *Caenorhabditis elegans* against oxidative and thermal stress. Food Chemistry **124:** 195-202.

Finan, T. M., Hirschj, A. M., Leigh, A., Johanseng, E., Kuldaus, A., Walker, D. C., and Signer, E. R. 1985. Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. Cell **40**: 869-877.

Fischer, H. M. 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. **58**: 352–386.

Fornes, F., Sanchez-Perales, M., and Guardiola, J. L. 2002. Effect of a seaweed extract on the productivity of "de nules" Clementine mandarin and Navelina orange. Bot. Mar. **45:** 486-489.

Fritig, B., Heitz, T., and Legrand, M. 1998. Antimicrobial proteins in induced plant defense. Curr. Opin. Immunol. **10**: 16-22.

Gage, D. J. 2002. Analysis of infection thread development using GFP- and DsRED-expressing *Sinorhizobium meliloti*. J. Bacteriol. **184**: 7042-7046.

Gage, D. J. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol. Mol. Biol. Rev. **68**: 280–300.

Galán Saúco, V. and Cubero, J.I. 2011. Contribution of Spain and Portugal to the exchange and acclimatization of new and old world crops. Acta Hort. (ISHS) **916:**71-82.

Galloway, J. N., Schlesinger, W. H., Levy II, H., Michaels, A., and Schnoor, J. L. 1995. Nitrogen fixation: anthropogenic enhancement – environmental response. Global Biochem. Cycles **9:** 235-252.

Galloway, J. N. 1998. The global nitrogen cycle: changes and consequences. Environmental Pollution **102:** 15-24.

Garau, G., Reeve, W. G., Brau, L., Deiana, P., Yates, R. J., James, D., Tiwari, R., O'Hara, G. W., and Howieson, J. G. 2005. The symbiotic requirements of different *Medicago* spp. suggest the evolution of *Sinorhizobium meliloti* and *S. medicae* with hosts differentially adapted to soil pH. Plant and Soil **176**: 263-277.

Gough, C. 2003. Rhizobium symbiosis: insight into nod factor receptors. Curr. Biol. **13**: 973-975.

Hartwig, U. A., Joseph, C. M. and Phillips, D. A. 1991. Flavonids released naturally from alfalfa seeds enhance growth rate of *Rhizobium meliloti*. Plant Physiol. **95:** 797-803.

Heidstra, R., Geurts, R., Franssen, H., Spaink, H. P., Van Kammen, A., and Bisseling, T. 1994. Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. Plant Physiol. **105:** 787-797.

Hirsch, A. M., and Fang, Y. 1994. Plant hormones and nodulation: what's the connection? Plant Mol. Biol. **26**: 5-9.

Hoagland, D. R., and Arnon, D. I. 1950. The water-culture method for growing plants without soil. California Agr. Exp. Sta. Circ. **347**:1-32.

Hong, D. D, Hien, H. M, Son, P. N. 2007. Seaweeds from Vietnam used for functional food,medicine and biofertilizer. J. Appl. Phycol. **19:** 817–826.

Honma, M. A., and Ausubel, F. M. 1987. *Rhizobium meliloti* has three functional copies of the *nod*D symbiotic regulatory gene. Proc. Natl. Acad. Sci. USA **84**: 8558-8562.

Ibragimova, M. V., Rumyantseva, M. L., Onishchuk, O. P., Belova, V. S., Kurchak, O. N., Andronov, E. E., Dzyubenko, N. I., and Simarov, B. V. 2006. Symbiosis between the root-nodule bacterium *Sinorhzobiummeliloti* and alfalfa (*Medicago sativa*) under salinization conditions. Microbiology **75:** 77-81.

Jo, J., Yoshida, S., and Kayama, R. 1980. Acidity tolerance and symbiotic nitrogen fixation capacity of some variaties of alfalfa. Nippon Sochi Gakkaishi, **26**: 174-178.

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., and Walker, G. C. 2007. How rhizobial symbionts invade plants: the Sinorhizobium–Medicago model. Nature Rev. Microbiol. **5**: 619-633.

Khan, W., Palanisamy, R., Hankins, S. D., Critchley, A. T., Smith, D. L., Papadopoulos, Y. A., and Prithiviraj, B. 2008. *Ascophyllum nodosum* (L.) Le Jolis extract improves root nodulation in alfalfa. Can. J. Plant Sci. **88**: 728-728.

Khan, W., Rayirath, U. P., Subramanian, S., Jithesh, M. N., Rayorath, P., Hodges, D. M., Critchley, A. T., Craigie, J. S., Norrie, J., and Prithiviraj, B. 2009. Seaweed Extracts as Biostimulants of Plant Growth and Development. J. Plant Growth Regul. **28:** 386-399.

Khan, W., Zhai, R., Souleimanov, A., Critchley, A. T., Smith, D. L., and Prithiviraj, B. 2012. A commercial extract of *Ascophyllum nodosum* improves root colonization of alfalfa by its bacterial symbiont *Sinorhizobium meliloti*. Comm. Soil Sci. Plant Anal. **43:** 1-12.

Kingman, A. R., and Moore, J. 1982. Isolation, purification and quantification of several growth regulating substances in *Ascophyllum nodosum* (Phaeophyta). Bot. Mar. **25**: 149-153.

Knops, J. M. H., Bradley, K. L., and Wedin, D. A. 2002. Mechanisms of plant species impacts on ecosystem nitrogen cycling. Ecology Letters **5:** 454-466.

Korsaeth, A., Bakken, L. R., and Riley, H. 2003. Nitrogen dynamics of grass as affected by N input regimes, soil texture and climate: Lysimeter measurements and simulations. Nutr. Cycl. Agroecosyst. **66:** 181‐199.

Lane, C. E., Mayes, C., Druehl, L. D., and Saunders, G. W. 2006. A multi-gene molecular investigation of the kelp (Laminariales, Phaeophyceae) supports substantial taxonomic re-organization. J. Phycol. **42**: 493-512.

Ledgard, S. F. 2001. Nitrogen cycling in low input legume‐based agriculture, with emphasis on legume/grass pastures. Plant and Soil **228:** 43-59.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J. C. and Denarie, J. 1990. Symbiotic host-specificity of Rhizobium meliloti is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344:** 781–784.

Liu Y., Wu L., Baddeley, J. A., and Watson, C. A. 2011. Models of biological nitrogen fixation of legumes. A review. Agron. Sustain. Dev**. 31:** 155-172.

Long, S. R. 1996. Rhizobium symbiosis: nod factors in perspective. Plant Cell **8:** 1885–1898.

McNeil, D. L. 2010. Biological nitrogen fixation in soybean. In: Singh, G. (ed) The soybean: botany, production and uses. Wallingford: CABI, 227-246.

Miller, J. H. 1972. Experiments in Molecular Genetics, published by Cold Spring. Harbor Laboratory Press. NY, 82, 6609-6613.

Mohammadi, K., Sohrabi, Y., Heidari, G., Khalesro, S., and Majidi, M. 2012. Effective factors on biological nitrogen fixation. Afr. J. Agr. Res. **7:** 1782-1788.

Mulder, L., Hogg, B., Bersoult, A., and Cullimore, J. V. 2005. Integration of signalling pathways in the establishment of the legume-rhizobia symbiosis. Physiol. Plant **123:** 207–218.

Mulder, L., Lefevre, B., Cullimore, J., and Imberty, A. 2006. Lysm domains of *Medicago truncatula* NFP protein involved in Nod factor perception. Glycosylation state, molecular modeling and docking of chitooligosaccharides and Nod factors. Glycobiology **16**: 801-809.

Mulligan, J. T., and Long, S. R. 1985. Induction of *Rhizobium meliloti nodC* expression by plant exudate requires *nodD*. Proc. Natl. Acad. Sci. USA **82**: 6609-6613.

Mulligan, J. T., and Long, S. R. 1989. A family of activator genes regulates expression of *Rhizobium meliloti* nodulation genes. Genetics **122**: 7-18.

Nabati, D. A., Schmidt, R. E., and Parrish, D. J. 1994. Alleviation of salinity stress in Kentucky bluegrass by plant growth regulators and iron. Crop Sci. **34:** 198-202.

Nelson, W. P., and Van Standen, J. 1984. The effect of seaweed concentrate on growth of nutrient-stresses, greenhouse cucumbers. Hortic. Sci. **19:** 81-82.

Oldroyd, G. E. and Downie, J. A. 2004. Calcium, kinases and nodulation signalling in legumes. Nature Rev. Mol. Cell Biol. **5**: 566–576.

Oldroyd, G. E., Engstrom, E. M., and Long, S. R. 2001. Ethylene inhibits the Nod factor signal transduction pathway of Medicago truncatula. Plant Cell **13:** 1835–1849.

Payne, R.W. 2002. The Guide to GenStat.Part 2: Statistics. VSN International Ltd, Oxford.

Perret, X., Staehelin, C., and Broughton, W. J. 2000. Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. **64**: 180–201.

Peters, N. K., Frost, J. W., and Long, S. R. 1986. A plant flavone, luteolin, induces expression of Rhizobium meliloti nodulation genes. Science **233:** 977–980.

Peterson, T. A., and Russelle, M. P. 1991. Alfalfa and the nitrogen cycle in the Corn Belt. J. Soil and Water Conservation **46:** 229-235.

Power, J. F., and Schepers, J. S. 1989. Nitrate contamination of groundwater in North America. Agriculture, Ecosystems and the Environment **26:** 165-187.

Prithiviraj, B, Paschke, M, and Vivanco, J. M. 2007. Root communication: the role of root exudates. Encycl. Plant Crop Sci. **1**:1-4.

Prithiviraj, B., Souleimanov, A., Zhou, X., and Smith, D. L. 2000. Differential response of soybean (*Glycine max* (L.) Merr.) genotypes to lipo-chitosaccharide Nod Bj V (C18:1 MeFuc). J. Exp. Bot.**51:** 2045–2051.

Rathore, S. S., Chaudhary, D. R., Boricha, G. N., Ghosh, A. Bhatt, B. P., Zodape, S. T., and Patolia, J. S. 2009. Effect of seaweed extract on the growth, yield and nutrient uptake of soybean (*Glycine max*) under rainfed conditions. S. Afr. J Bot. **75:** 351-355.

Rayorath, P., Jithesh, M. N., Farid, A., Khan, W., Palanisamy, R., Hankins, S. D., Critchley, A. T., and Prithiviraj, B. 2008a. Rapid bioassays to evaluate the plant growth promoting activity of *Ascophyllum nodosum* (L.) Le Jol. using a model plant, *Arabidopsis thaliana* (L.) Heynh. J. Appl. Phycol. **20**: 423-429.

Rayorath, P., Khan, W., Palanisamy, R., MacKinnon, S. L., Stefanova, R., Hankins, S. D., Critchley, A. T., and Prithiviraj, B. 2008b. Extracts of brown seaweed, *Ascophyllum nodosum*, induce gibberellic acid (GA3) independent amylase activity in barley. J. Plant Growth Regul. **27**:270-279.

Rayorath, P, Benkel, B, Hodges, M. D, Allan-Wojtas, P., MacKinnon, S., Critchley, A. T., and Prithiviraj, B. 2009. Lipophilic components of brown seaweed, *Ascophyllum nodosum*, enhance freezing tolerance in Arabidopsis thaliana. Planta **230:**135-147.

Reitz, S. R., and Trumble, J. T. 1996. Effects of cytokinin-containing seaweed extract on *Phaseolus lunatus* L.: Influence of nutrient availability and apex removal. Bot. Mar. **39:**33–38.

Relic, B., Perret, X., Estrada-Garcia, M. T., Kopcinska, J., Golinowski, W., Krishnan, H.B., Pueppke, S.G. and Broughton, W.J. 1994. Nod factors of Rhizobium are a key to the legume door. Mol. Microbiol. **13:** 171–178.

Relid, B., Talmont, F., Kopcinska, J., Golinowski, W., Prome, Broughton, W. J. 1993. Biological activity of *Rhizobium* sp. NGR234 Nod-factors on *Macroptilium atropurpureum*. Mol. Plant-Microbe Interact. **6**: 764-774.

Ritchie, M. E., Tilman, D., and Knops, J. 1998. Herbivore effects on plant and nitrogen dynamics in oak savanna. Ecology **79:** 165–177.

Sanon, A., Andrianjaka, Z. N., Prin, Y., Bally, R., Thioulouse, J., Comte, G., and Duponnois, R. 2009. Rhizosphere microbiota interfers with plant-plant interactions. Plant Soil **321**: 259-278.

Sathya, B., Indu, H., Seenivasan, R., and Geetha, S. 2010. Influence of seaweed liquid fertilizer on the growth and biochemical composition of legume crop, *Cajanus cajan* (L.) Mill sp. J. Phytol. **2:** 50-63.

Schmidt, I. K., Michelson, A., and Jonasson, S. 1997. Effects of labile soil carbon on nutrient partitioning between an arctic graminoid and microbes. Oecologia **112:** 557–565.

Seigler, D. S. 1998. Plant Secondary Metabolism, Kluwer Academic Publishers.

Schimel, J. P., and Bennett, J. 2004. Nitrogen mineralization: Challenges of a changing paradigm. Ecology **85:** 591 ‑ 602.

Silva, C., Kan, F. L., and Mart´ınez-Romero, E. 2007. Population genetic structure of *Sinorhizobium meliloti* and *S.medicae* isolated from nodules of *Medicago* spp. In Mexico. FEMS Microbiol. Ecol. **60**: 477-489.

Small, E., and Jomphe, M. 1989. A Synopsis of the genus Medicago. Can. J. Bot. **67**: 3260-3294.

Smil, V. 2001. Enriching the Earth: Fritz Haber, Carl Bosch, and the transformation of world food production. MIT Press, Cambridge, Mass.

Smit, P. 2007. Nod factor signaling and infection in *Rhizobium*-legume symbiosis. Ph.D. thesis, Wageningen University, The Netherlands.

Sprent, P., and Sprent, J. I. 1990. Nitrogen fixing organisms: Pure and applied aspects. 1st ed. University Press, Cambridge. 256 pp.

Statistic Canada. 2006. Census of Agriculture counts 3,795 farms in Nova Scotia. http://www.statcan.gc.ca/ca-ra2006/analysis-analyses/ns-ne-eng.htm

Steinkellner, S., Lendzemo, V., Langer, I., Schweiger, P., Khaosaad, T., Toussaint, J. P., and Vierheilig, H. 2007. Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic Plant-Fungus interactions. Molecules **12**: 1290-1306.

Sunarpi, A. J., Kurnianingsih, R., Julisaniah, N. I., and Nikmatullah, A. 2010. Effect of seaweed extracts on growth and yield of rice plants. Bioscience **2**: 73-77.

Temple, W. D., and Bomke, A. A. 1989. Effects of Kelp (*Macrocystis integrifolia* and *Eklonia maxima*) foliar applications on bean crop growth. Plant Soil **117**: 85-92.

Vance, C. P. 1998. Legume symbiotic nitrogen fixation: agronomic aspects. In: Spaink HP, Kondorosi A, Hooykaas PJJ (ed) The *Rhizobiaceae*. Kluwer, Dordrecht, pp 509–530.

Verkleij, F. N. 1992. Seaweed extracts in agriculture and horticulture: a review. Biol. Agric. Hortic. **8:** 309–324.

Vincent, J. M. 1970. A manual for the practical study of root nodule bacteria, 73–79. Oxford, UK: International Biological Programme Handbook, Blackwell Scientific Publications.

Wagner, S. C. 2011. Biological Nitrogen Fixation. Nature Education Knowledge **2**:14.

Webb, K. T. and Langille, D. R. 1995. Soils of the Nappan Research Farm, Nova Scotia. Centre for Land and Biological Resources Research, Research Branch, Agriculture and Agrifood Canada, [Truro, N.S.]

Webb, K. T. 1991. Soils of Colchester County, Nova Scotia. Research Branch, Agriculture Canada, [Ottawa].

Weir, B. S. 2012. The current taxonomy of rhizobia. New Zealand rhizobia website. http://www.rhizobia.co.nz/taxonomy/rhizobia.html. Last updated: 10th April, 2012.

Zhang, X., Eirvin, E. H. 2004. Cytokinin-containing seaweed and humic acid extracts associated with creeping bentgrass leaf cytokinins and drought resistance. Crop Science **44:** 1737-1745.

Appendix: Field ANOVA Table

Treatment	DM Yield t ha^{-1}		# Of Functional Nodule		Root Length cm		Leaf Area cm^2		Root DW g		Shoot DW g	
	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010
Control	2.20	4.60	16	13	110.12	124.93	92.97	124.90	0.29	1.89	0.85	3.95
0 g L^{-1}	2.22	4.35	15	10	102.30	173.48	73.48	210.00	0.35	1.86	0.90	3.88
1 g L^{-1}	2.43	4.77	16	14	97.05	170.93	88.76	291.03	0.32	1.71	0.86	3.84
2 g L^{-1}	2.29	4.68	17	14	110.33	171.42	95.81	233.40	0.32	1.69	0.95	3.74
3 g L^{-1}	2.25	4.89	16	12	99.42	212.20	80.28	263.17	0.28	1.81	0.79	4.88
4 g L^{-1}	2.35	5.37	16	12	93.90	147.53	92.71	158.77	0.34	1.89	1.09	4.96
Mean	2.29	4.78	16	13	102.19	166.75	87.34	213.54	0.32	1.81	0.91	4.21
SEM	0.161	0.423	1.120	0.954	10.575	11.445	7.967	27.157	0.030	0.183	0.089	0.704
F prob. Ctrl VS SWT	NS	NS	NS	NS	NS	0.001	NS	0.002	NS	NS	NS	NS
Lin SWT	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Quad SWT	NS	NS	NS	0.010	NS	NS	NS	0.011	NS	NS	NS	NS