Jasmonates as a New Class of Signaling Molecules in *Bradyrhizobium*-Soybean Symbiosis

By

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Jasmonates as signal molecules in *Bradyrhizobium*-soybean symbiosis

DEDICATION

To my parents, without whose numerous sacrifices
I would probably never have started writing

ABSTRACT

Fazli Mabood

Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), collectively termed as jasmonates, are naturally occurring in plants and are important signal molecules involved in induced disease resistance and stress responses of plants. Besides their role *in-planta*, they are also rhizosecreted by root cells. Germinating soybean seeds exude large quantities of jasmonic acid; however there is no knowledge regarding how jasmonates influence cells of the soybean symbiont, *Bradyrhizobium japonicum,* in the rhizosphere. We studied the role of jasmonates in the soybean-*Bradyrhizobium* symbiosis. *Bradyrhizobium japonicum* cultures were induced with jasmonates and the resulting Nod factors were isolated and purified. Our results showed that JA and MeJA strongly induced the production of Nod factors by the tested *B. japonicum* strains. When added together, genistein and jasmonates resulted in greater LCO production than either one alone. Jasmonic acid is produced from linoleic and linolenic acids via the octadecanoid pathway; we studied the effect of these two fatty acids on *B. japonicum nod* gene induction. Interestingly both linoleic and linolenic acids induced the *nod* genes and caused LCO production by *B. japonicum* cultures. Since jasmonates induced the *nod* genes and also caused LCO production in *B. japonicum*, I conducted experiments in the greenhouse and field to determine whether incubation of *B. japonicum* with JA or MeJA prior to inoculation increases soybean nodulation variables and grain yield. Both genistein and MeJA increased nodule number and nodule dry weight per plant. Due to enhanced nitrogen fixation, attributed to increased nodule number and weight, soybean dry matter accumulation and grain yield were increased. These results document the discovery of jasmonates and their precursors as new signal molecules in the *Bradyrhizobium* – soybean nitrogen fixing symbiosis.

RÉSUMÉ

L'acide jasmonique (AJ) et son ester méthylique, le jasmonate méthylique (JAMe), collectivement dénommés comme jasmonates, se retrouvent naturellement chez les végétaux. Ces deux composés sont des molécules importantes de signal impliqués dans la résistance induite contre les maladies et participe aux réponses de stress dans les plantes. En plus de leur rôle *in-planta*, ils sont secretées également par des cellules de la racine. Lors de la germination les graines de soja libèrent de grandes quantités d'acide jasmonique; toutefois nous ne connaissons pas par quel moyen les jasmonates influencent les cellules du symbiont de soja, le *Bradyrhizobium japonicum*, dans la rhizosphère. Nous avons étudié le rôle des jasmonates dans la symbiose de soja-*Bradyrhizobium*. Des cultures de *Bradyrhizobium japonicum* ont été induites avec des jasmonates et les facteurs Nod résultant de l'induction ont été isolés et purifiés. Les résultats provenant des souches de *B. japonicum* testées ont prouvé que JA et MeJA induisent fortement la production des facteurs Nod. Lorsque combinés, le genistein et les jasmonates ont eu comme conséquence une plus grande production de LCOs que l'application d'un seul de ces composés. L'acide jasmonique est produit à partir des acides linoléiques et linoléniques par l'intermédiaire de la voie octadécanoidale; nous avons étudié l'effet de ces deux acides gras sur l'induction des gènes *nod* de *B. japonicum*. Il est intéressant de noter que les acides linoléiques et linoléniques ont activé les gènes *nod* et la production des LCOs au moyen de cultures de *B. japonicum*. Puisque les jasmonates ont activé les gènes *nod* et la production des LCOs chez le *B. japonicum*, j'ai fait des expériences en serre et en champs pour déterminer si l'exposition de *B. japonicum* a l'AJ ou l'JAMe avant inoculation mènerait a une augmentation de la nodulation du soja et du rendement . Le génisteine et le JAMe ont augmenté le nombre des nodules et le poids sec des nodules par plante. En raison de la fixation augmentée de l'azote, attribuée a une augmentation du nombre et du poids des nodules, l'accumulation de matière sèche de soja et le rendement de grain ont été améliorés. Ces résultats supportent la découverte que les jasmonates et leurs précurseurs sont des nouvelles molécules de signal dans la symbiose entre *Bradyrhizobium* et le soja.

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CONTRIBUTIONS OF CO-AUTHORS TO MANUSCRIPTS FOR PUBLICATION

Based upon statement from the thesis office, this thesis has been written in the form of manuscripts which have been submitted to scientific journals. Thus, the contents of sections 3 to 6 inclusively are drawn from the manuscripts for publication.

The manuscripts from which sections 3 and 4 are taken were co-authored by myself, Alfred Souleimanov and Donald L. Smith. Dr. Donald Smith, my supervisor at the Macdonald Campus of McGill University, provided funds, assistance and supervisory guidance from the outset of the research to the reviewing of the manuscripts before submission for publication. He was also responsible for arranging laboratory, growth benches, experimental field spaces, the use of computers, and providing technical assistance. Dr. Souleimanov is a Research Associate in Dr. Smith's laboratory. He helped me in conducting the HPLC analyses for my experiments. The contribution of Dr. Smith to this manuscript was as described previously.

The manuscript from which section 5 is taken is written by myself and Donald Smith. The contribution of Dr. Smith to this manuscript was as described previously.

The manuscript from which section 6 is taken was co-authored by myself, Xiaomin Zhou and Donald L. Smith. Dr. Xiaomin Zhou is a Research Associate and laboratory manager in Dr. Smith's laboratory. She helped in designing the experiment and arranging laboratory and field equipment. The contribution of Dr. Smith to this manuscript was as described previously.

Section 1
GENERAL INTRODUCTION

1.1. Introduction

Legume plants have unique organs, nodules, on their roots and sometimes on stems wherein rhizobia reside in a differentiated form, the bacteroid, and fix nitrogen by reducing atmospheric dinitrogen into ammonia. Biological nitrogen fixation (BNF) is of primary importance for introduction of fixed N into the biosphere and is estimated to occur in the range of 40-48 million tonnes per year (Gallo-way *et al.*, 1995; Jenkinson, 2001). Industrial N fixation, applied to the crops in the form of fertilizers, accounts for up to 83 million tonnes of N per year (Jenkinson, 2001). BNF is environmentally friendly and sustainable as it is driven by solar energy trapped by crop plants in the form of photosynthesis (Bohlool *et al.*, 1992). Soybean [*Glycine max* (L.) Merr] plants, by forming a symbiosis with the bacterium *Bradyrhizobium japonicum*, can fix up to 200 kg N ha^{-1} $year^{-1}$ of atmospheric N_2 under optimal environmental conditions (Smith and Hume, 1987).

The formation of a nitrogen-fixing soybean nodule is a complex and fine-tuned process that requires the production and successful exchange of specific molecular signals between micro- and macrosymbionts. Soybean roots exude isoflavonoids, some of which can act as plant-to-*Bradyrhizobium* signals. The most effective of soybean plant-to-bacteria signal molecules is the isoflavone genistein (Kosslak *et al.*, 1987) which induces *Bradyrhizoboium japonicum nod* (including *nod, noe* and *nol*) genes by binding to regulatory proteins (NodD1, NodVW, and/or NolA). The products of the *nod* genes are Nod factors, structurally identified as lipo chitooligosaccharides (Carlson *et al.*, 1993). Nod factors mediate many of the early steps leading to the organogenesis of nitrogen fixing nodules (Sanjuan *et al.*, 1992; Stacey *et al.*, 1995).

Soybean is a subtropical legume and for optimum plant growth and symbiotic activity requires soil temperatures from 25 to 30 °C (Jones and Tisdale, 1921). Soybean nodule establishment and nitrogen fixation activity declines when the soil temperature drops below this range. Previous studies in our laboratory have established that low soil

temperatures decrease both nodulation and nitrogen fixation by soybean plants. The onset of nitrogen fixation in soybean plants is delayed by 2-3 days for each degree decrease in soil temperature when the soil temperature drops below 25 °C but remains above 17 °C. Further decrease in soil temperature, from 17 to 15 °C, is more inhibitory and delays the onset of nitrogen fixation by 1 week for each degree Celsius (Zhang and Smith, 1994, Zhang *et al.,* 1995). However, nodulation fails when the plants are grown at 10 °C (Matthews and Hayes 1982). The early steps of the *Bradyrhizobium*-soybean symbiosis are most sensitive; low soil temperature disrupts the inter-organismal signal exchange that occurs prior to nodule primordial initiation (Zhang and Smith, 1995). Low soil temperature decreases both the biosynthesis and rhizosecretion of genistein from soybean plant root cells into the soil rhizosphere (Zhang and Smith, 1996a). It also inhibits the induction of *nod* genes of *B. japonicum* (Zhang *et al.,* 1996). All stages of symbiotic establishment investigated to date (root hair curling, infection thread formation and penetration, nodule development and function) are inhibited by suboptimal temperatures (less than 25°C) (Zhang and Smith, 1994).

In higher latitude regions such as Canada, low soil temperature is considered to be the major factor potentially limiting soybean growth and symbiotic nitrogen fixation (Whigam & Minor, 1978). In order to reduce or partially overcome the negative effects of low-soil-temperature-induced inhibitory effects on soybean nodulation, previous studies from our laboratory have shown that the addition of genistein pre-induced rhizobia to the soybean seeds at the time of seeding or application of genistein directly into soil has proven to be an effective means of enhancing nodulation and nitrogen fixation leading to increased soybean yield (Zhang and Smith, 1996b; 1997). This "SoyaSignal" technology mainly focuses on enhanced *Bradyrhizobium*-soybean inter-organismal signaling and has been patented in the US (Smith and Zhang, 1996).

The genistein based "SoyaSignal" technology has now been in the market place for several years. However, the use of flavonoids in commercial production presents two challenges. First, they are very expensive (on the order of \$100 g^{-1}). Second, flavonoids often act as phytoallexins in plant responses to pathogenic attacks (Dixon *et al.*, 1995; Dakora and Phillips, 1996) and at the higher concentrations of genistein (20 μM) required

to induce *B. japonicum* cells at low temperatures they slow the development of *B. japonicum* cultures, adding cost to commercial production practices.

In view of these circumstances, it would be valuable to find an alternative to the flavonoid-based-technology. Research efforts focused on identifying compounds that can induce *nod* genes, cause Nod factor production, enhance nodulation and nitrogen fixation, are not deleterious to rhizobial cells at concentrations applied to the seeds and are less expensive than genistein would be of immense interest to the end users and would serve to promote soybean production in cooler agricultural regions of the world. In addition, improved understanding of the signaling that occurs between plants and soil microbes, in this case legumes and rhizobia, will expand our knowledge of what is proving to be a fascinating area.

1.2. Hypotheses

The purpose of this study was to search for new *nod* gene inducers for the *Bradyrhizobium*-soybean symbiosis; elucidate the mechanism and role of potential *nod* gene inducer compounds on the induction of *nod* genes in *B. japonicum* and to devise a signal-compound-based-technology that reduces the negative effects of suboptimal root zone temperatures (RZTs) on the soybean N_2 fixing symbiosis under cool spring conditions, such as those prevalent in much of the Canadian soybean production region. Therefore, this research project had the following research hypotheses:

1) *Bradyrhizobium japonicum* cultures, when induced with jasmonates, produce and secrete Nod factors (lipo-chitooligosaccharides - LCOs).
2) The fatty acid precursors of jasmonates, linolenic and linoleic acids, also induce *nod* gene expression and cause Nod factor production by *B. japonicum*
3) Jasmonates pre-incubated bacterial cultures of *B. japonicum* enhance nodulation and nitrogen fixation at suboptimal RZTs under greenhouse conditions
4) Jasmonate pre-incubated bacterial cultures of *B. japonicum* enhance nodulation, nitrogen fixation, plant growth and grain yield of soybean under short season field conditions

1.3. Objectives

The objectives of this thesis were:

1. To determine whether or not *B. japonicum* cultures induced with jasmonates produce and secrete Nod factors (lipo chitooligosaccharides - LCOs).
2. To investigate whether or not the octadecanoid pathway fatty acid precursors, linolenic and linoleic acids, can induce *nod* genes and cause Nod factor production by *B. japonicum*
3. To determine whether or not jasmonate pre-incubated bacterial cultures of *B. japonicum* can enhance nodulation and nitrogen fixation at suboptimal RZTs under greenhouse conditions
4. To determine whether or not jasmonate pre-incubated bacterial cultures of *B. japonicum* can enhance nodulation, nitrogen fixation, plant growth and grain yield of soybean under short season field conditions

NOTE: I did not formulate all these objectives at the start of my Ph D research. Some of the objectives were formulated following findings of the experiments conducted during the first part of my research.

2. LITERATURE REVIEW

2.1. Soybean and *Bradyrhizobium japonicum*

Soybean (*Glycine max* (L.) Merr.) is one of the world's most economically important legume crops. It combines in one crop both the dominant world supply of edible vegetable oil, and the dominant supply of high-protein feed supplements for livestock. Other fractions and derivatives of the seed have substantial economic importance in a wide range of industrial, food, pharmaceutical, and agricultural products (Smith and Huyser, 1987).

Soybean [*Glycine max* (L.) Merr.] belongs to the family fabaceae (Leguminoseae), subfamily Papilionideae. The cultivated species of soybean is a summer annual. Soybean is thought to have originated in central China, as early as 2800 B.C. (Smith, 1995). Because of its tropical to subtropical origins, soybean needs an optimum temperature range of 25-30 ^{o}C for normal growth and development (Jones and Tisdale, 1921). Legume plants are unique in that they are able to form specific symbioses with soil born rhizobia. Nodules are the special plant organs that develop on roots and sometimes on stems of the legume plants where rhizobia reside and can fix atmospheric atmospheric dinitrogen. This fixed nitrogen is then assimilated by the plant to support plant growth and protein production (Long, 1989). Soybean and *Bradyrhizobium japonicum* are two such partners, able to establish a nitrogen fixing symbiosis (Stacey *et al.,* 1995).

The genus *Bradyrhizobium* consists of two species, *B. japonicum* and *B. elkanii,* as well as some other un-named strains (*B.* spp.*)*. Bradyrhizobia are aerobic, gram negative rod shaped, 0.5-0.9 μm x 1.2-3.0 μm in size, non-spore forming, motile through one or two polar or sub-polar flagella with a high G + C content (from 61 to 65%). The colonies are circular, white, opaque, convex and granular in structure. They are slow-growing on artificial media with generation time more than 8 hours; have a T_{opt} of 25 – 30^{o}C and a pH_{opt} of 6.0 – 7.0. The diameter of the colony doesn't exceeds 1 mm after 5-7 days of incubation on yeast extract mannitol (YEM) agar plates and broth cultures only develop a moderate turbidity after shaking for 3-5 days. Bradyrhizobia have an alkaline reaction on YEM and are not able to use disaccharides as a carbon source (Krieg, 1984).

Bradyrhizobium japonicum are able to persist as free living in soils. They are able to infect and induce nodules on soybean (*Glycine max* and *G. soja*), cowpea (*Vigna unguiculata*), mungbean (*Vigna radiata*) and siratro (*Macroptilium atropurpureum*) root systems. Inside the root nodules, they reside as bacteroids and can fix atmospheric dinitrogen which is then utilized by the host plant (Stacey *et al.,* 1995).

2.2. Soybean nodulation and nitrogen fixation

Soybean has the ability to establish symbiotic interactions with soil bacteria *Bradyrhizobium japonicum*. The establishment of this symbiotic system, the nodulation process, is a result of a complex process consisting of a series of interactions between the host and the bacteria. The earliest event during this association is signal exchange between two symbionts. Both symbionts produce and exchange diffusible signal molecules followed by attachment of the bacteria to the host plant root hairs. The root hair curls and nearly encircles the bacteria. The bacteria invade the plant root hair via an infection thread formed inside the root hairs. Upon reaching the cortical cells, the bacterial signals induce cortical cell division leading to the ontogenesis of nodule primordia. The bacteria are released into the cytoplasm of the host plant cells. The nodule primordium develops into a mature nodule, while the bacteria differentiate into their endosymbiotic forms as bacteroids. At this stage, the bacteroids produce dinitrogenase, which catalyzes the reduction of atmospheric dinitrogen to produce ammonia, fulfilling plant nitrogen demand and supporting plant protein production (reviewed by Mylona *et al.*,1995).

2.2.1. Plant-to-bacteria signaling molecules: The biosynthesis and release of (iso)flavonoid molecules

Flavonoids are a diverse group of phenolic compounds and serve a variety of ecological and physiological functions in plants (Stafford, 1990; Mathesius *et al.,* 1998a; Debeaujon, *et al.,* 2000). Flavonoids occur ubiquitously in plants and more than 4000 flavonoids have been characterized (Perret *et al.,* 2000). Flavonoids are the best understood class of *nod*

gene inducer molecules in rhizobia-legume symbioses, and their role in *nod* gene induction has been widely studied (Gottfert, 1993, Perret *et al.,* 2000). They can be classified into subgroups such as flavanols, flavonols, flavones, flavanones, and isoflavonoids. While flavonoids are found throughout the plant kingdom, isoflavonoids are limited to the legume family (Perret *et al.*, 2000). In plants flavonoids are biosynthesized from the precursor amino acid phenylalanine, which is a product of the shikimic acid pathway. Phosphoenolpyruvic acid is produced through glycolysis and it combines with D-erythose-4-phosphate, produced from the pentose phosphate pathway, leading to the formation of shikimic acid. Shikimic acid is further modified, leading to the biosynthesis of tyrosine, tryptophan, and phenylalanine. Phenylalanine acts as a precursor of the phenylproponoid pathway. This pathway is essentially an extension of the shikimic acid pathway, creating secondary metabolites in the presence phenylalanine ammonia lyase (PAL). Phenylalanine is converted into trans-cinnamic acid in the presence of PAL, the first committed step in this pathway. Cinnamic acid is the starting point of many secondary metabolites, producing condensed tannins, flavonoids, lignins, and simple phenolic compounds (Figure 2.1).

The biosynthesis of isoflavonoids from the precursor L-phyenylalanine can be viewed as occurring via 3 sub-pathways: 1) the core phenylproponoid pathway, consisting of enzymatic reactions from phenylalanine to 4-coumaroyl coenzyme A, (CoA); 2) the flavonoid synthesis pathway comprised of steps leading from the conversion of 4-coumaroyl CoA and malonyl CoA to a flavanone intermediate; and 3) the isoflavonoid-specific branch pathway leading to the biosynthesis of isoflavonoids from the flavanone intermediate molecules.

The conversion of L-phenylalanine (Phe) into trans-cinnamic acid due to the action of phenylalanine ammonia lyase (PAL; EC 4.3.1.5) is the first committed step in the biosynthesis of flavonoids and related compounds. PAL is a tetrameric enzyme and, in most species, its subunits are encoded by a multigene family (Cramer *et al.,* 1989; Nagai *et al.,* 1994; Wanner *et al.,* 1995; Fukasawa-Akada *et al.,* 1996). The second step in the phenylpropanoid pathway is the hydroxylation of trans-cinnamic acid into 4-coumaric acid, which is catalyzed by cinnamic acid 4-hydroxylase (C4H; EC 1.14.13.11), a cytochrome P450 monooxygenase enzyme (Russell and Conn, 1967; Fahrendorf and

Dixon, 1993; Teutsch *et al.* 1993). The induction of C4H by light, elicitors, and wounding have all been investigated (Fahrendorf and Dixon, 1993; Buell and Somerville, 1995; Batard *et al.,* 1997; Bell-Lelong *et al.,* 1997), and is closely coordinated with PAL induction (Mizutani *et al.*, 1997).

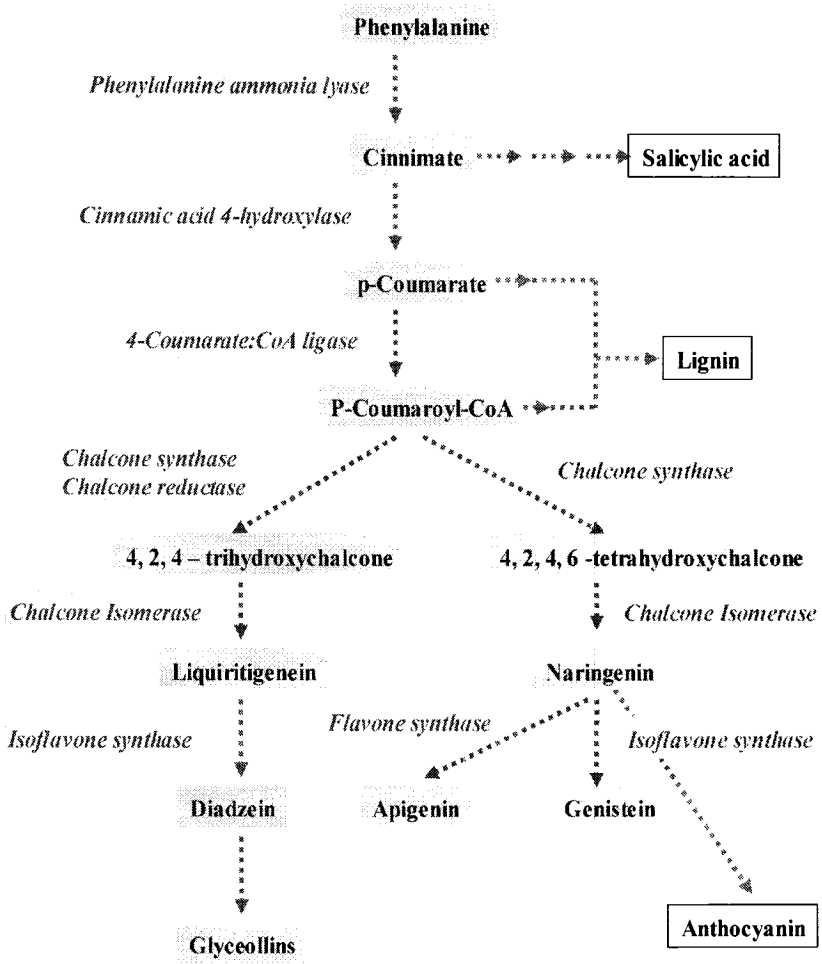
Three moles of malonyl-coenzyme A (CoA) from glucose metabolism condense to form ring A, catalyzed by chalcone synthase (Figure 2.1). Rings B and C also come from glucose metabolism, but via the shikimate pathway, through phenylalanine, which is converted to cinnamic acid and then to coumaric acid. Coumaric acid CoA and three malonyl CoAs condense through a single enzymatic step to form naringenin chalcone. The C-ring closes and becomes hydrated to form 3-hydroxyflavonoids (e.g. catechins), 3,4-diol flavonoids (e.g. quercetin), and procyanidins (Formica and Regelson, 1995).

The isoflavone biosynthetic pathway is a specialized branch pathway restricted to legumes. The enzyme isoflavone synthase catalyzes the first reaction specific to isoflavonoid biosynthesis. The mechanism responsible for migration of an aryl group from the C2 to the C3 position of the flavanone substrate is not fully understood, however it is believed that it involves a two step reaction initiated by a P450-dependent hydroxylation of the 2 or 3 position of the flavanone substrate (Kochs and Grisebach, 1989). After migration of aryl group, the 2-hydroxyisoflavanone intermediate undergoes a dehydration to yield the corresponding isoflavone (Hakamatsuka *et al.,* 1998). Genistein (4', 5, 7-trihydroxyisoflavone) is the product of aryl migration/dehydration of naringenin, whereas diadzein (4', 7-dihydroxyisoflavone) is formed in a similar fashion from liquiritigenin (4', 7-dihydroxyflavanone) (Yanagihara *et al.*, 1993). Isoflavone synthase catalyzes both these reactions, leading to the biosynthesis of diadzein and genistein.

Flavonoid storage in the plant cell vacuoles occurs through glycosylation or malonylation. Generally, the sugar moieties that form the flavonoid glucosides are removed through hydrolysis before the aglycone forms are exuded into the rhizosphere, however, glycoside forms of flavonoids are sometimes found in the rhizosphere (Leon-Barrios *et al.,* 1993). Studies have shown that the flavonoid moiety is the active *nod* gene inducer for both aglycone and glycoside forms (Hungria *et al.,* 1991). However, the glucoside forms of flavonoids sometimes lose their *nod* gene induction activity. The alfalfa seed coat possesses high levels of luteolin-7-O-glucoside, which is not active in

nod gene induction, however the aglycone form luteolin is active (Hartwig and Phillips, 1991). Soybean produces two major *nod* gene inducing isoflavones -: genistein and diadzein. Genistein is a stronger inducer of *nod* genes in *Bradyrhizobium japonicum* than diadzein (Kosslak *et al.,* 1987).

Figure 2.1. The biosynthesis of flavonoids via phenylpropanoid pathway.
The biosynthesis of flavonoids is mediated via the phenylpropanoid pathway. Phenylalanine is converted into cinnamic acid through a series of intermediates, leading to the biosynthesis of flavonoids. The key enzymes in this pathway are CH (cinnamic acid hydroxylase), CHS (chalcone Synthase), CHR (chalcone Reductase), CHI (chalcone isomerase), IS (isoflavone synthase), isoflavanone synthase and flavone synthase. The red lines show the pathway leading to the biosynthesis of flavonoids and iso-flavonoids. Compounds of this pathway are shown in shaded boxes, while the enzymes of this pathway are italicized. The branch pathways compounds are in boxes with a white background (adapted and modified from Yu *et al.*, 2000).


Phenylalanine
Phenylalanine ammonia lyase
Cinnimate
Salicylic acid
Cinnamic acid 4-hydroxylase
p-Coumarate
4-Coumarate:CoA ligase
Lignin
P-Coumaroyl-CoA
Chalcone synthase
Chalcone reductase
Chalcone synthase
4, 2, 4 – trihydroxychalcone
4, 2, 4, 6 -tetrahydroxychalcone
Chalcone Isomerase
Chalcone Isomerase
Liquiritigenein
Naringenin
Flavone synthase
Isoflavone synthase
Isoflavone synthase
Diadzein
Apigenin
Genistein
Anthocyanin
Glyceollins

2.2.2. Transcriptional regulation of *nod* genes in *Bradyrhizobium japonicum*

a) The NodD model

The bacterial nodulation genes (*nod, nol, noe*) are involved in the biosynthesis of Nod factors necessary for the establishment of nitrogen fixing nodules (Day *et al.,* 2000; Spaink, 2000). These *nod* genes are expressed specifically in response to plant-produced flavonoid compounds. In the presence of specific plant-to-bacteria signal molecules, *nod* gene regulation is mediated by NodD, a LysR-type regulator (Gottfert *et al.,* 1990; Kosslak, *et al.,* 1987). The NodD protein binds to the nod box sequence upstream of the *nod* genes (Fisher *et al.,* 1988; Goethals *et al.,* 1992; Hong *et al.,* 1987) and induces DNA bending (Fisher and Long, 1993; Fisher and Long, 1989), leading to transcriptional activation upon recognition of the inducer. In addition, the presence of flavonoids also increases binding of NodD to the *nod* gene promoter region (Yeh *et al.,* 2002).

The number of *nodD* genes varies among rhizobia. *Sinorhizobium meliloti* has 3 copies of *nodD* (Honma *et al.,* 1990) while *Rhizobium tropici* has 5 copies of the *nodD* gene (van Rhijn *et al.,* 1993). In *S. meliloti* these NodD proteins respond to different groups of flavonoids, suggesting that NodD redundancy allows the bacterium to infect multiple hosts secreting a wide range of flavonoids (Kondorosi *et al.,* 1991; Mulligan and Long, 1989).

In *Bradyrhizobium japonicum* two NodD proteins (NodD1 and NodD2) were identified. They have different expression patterns and functions. NodD1 responds to isoflavones, genistein and diadzein, and acts as a positive transcriptional activator (Banfalvi, *et al.,* 1988; Gottfert *et al.,* 1990; Kosslak *et al.,* 1987). Alternatively, NodD2 is involved in *nod* gene repression (Garcia *et al.,* 1996; Gillette and Elkan, 1996) (Figure 2.2.). *B. japonicum* NodD1 is autoregulated and is induced by genistein and daidzein, a phenomenon that is different from the constitutive expression of NodD found in most *Rhizobium sp.* (Smit *et al.,* 1992). This induction is mediated by a divergent nod box sequence 5' of the *nodD1* gene (Wang and Stacey, 1991). In addition to genistein and daidzein, nodD1 is also induced by conjugated isoflavones (e.g., 6-O-malonyldaidzin and 6-O-malonyl genistin) (Smit *et al.,* 1992). These conjugated derivatives are unable to

induce the expression of *nodYABC* transcription, thus providing the plant the potential to specifically regulate nodD1 expression.

It is generally perceived that the NodD remains the central regulator in *nod* gene regulation; however, additional regulators are now known to modulate the induction of the *nod* genes. For instance, in *S. meliloti* a second LysR type regulator, SyrM, regulates *nod* gene expression in a flavonoid-independent fashion (Kondorosi *et al.,* 1991; Maillet *et al.,* 1990). In *B. japonicum*, it is also mediated by the members of two other families (i.e., two-component regulators *nod* VW and a MerR-type regulator NolA) (Loh and Stacey, 2003).

b) NodD and beyond: two-component regulation

The regulation of *nod* genes in *B. japonicum* is further complicated in that it requires other regulatory proteins than just nodD1. This was discovered when *B. japonicum* nodD1 mutants were still able to nodulate host plants (Gottfert *et al.,* 1990). Subsequent findings showed that NodVW are also involved in *nod* gene regulation (Sanjuan *et al.,* 1994). Originally identified as a host-specific gene by Göttfert *et al.* (1990), NodVW is essential for the nodulation of cowpea, siratro, and mungbean, but not soybean. One possible explanation for the host-specific function of NodVW may be that it specifically recognizes flavonoids produced by cowpea, siratro, and mungbean, but not soybean. This way, it provides *B. japonicum* the flexibility to nodulate a broader range of plants. Alternatively, the infection of cowpea, siratro, and mungbean may demand higher levels of Nod signal production than needed for soybean infection. The combined efforts of NodD and NodVW would be necessary for increased Nod signal synthesis.

NodV and NodW are members of the two-component regulatory family (Gottfert *et al.,* 1990; Stock *et al.,* 2000) (Figure 2.2). On detection of an environmental stimulus, autophosphorylation of the sensor kinase (NodV) occurs with subsequent transfer of the phosphoryl group to its regulator protein (NodW) (Loh *et al.,* 1997). The phosphorylated protein is then primed to activate its target genes (*nodYABC* operon of *B. japonicum*). Phosphorylation of NodW, which likely occurs at the Asp70 residue in response to the isoflavonoid genistein, is critical for *nod* gene expression and nodulation.

c) Repression of the *nod* genes

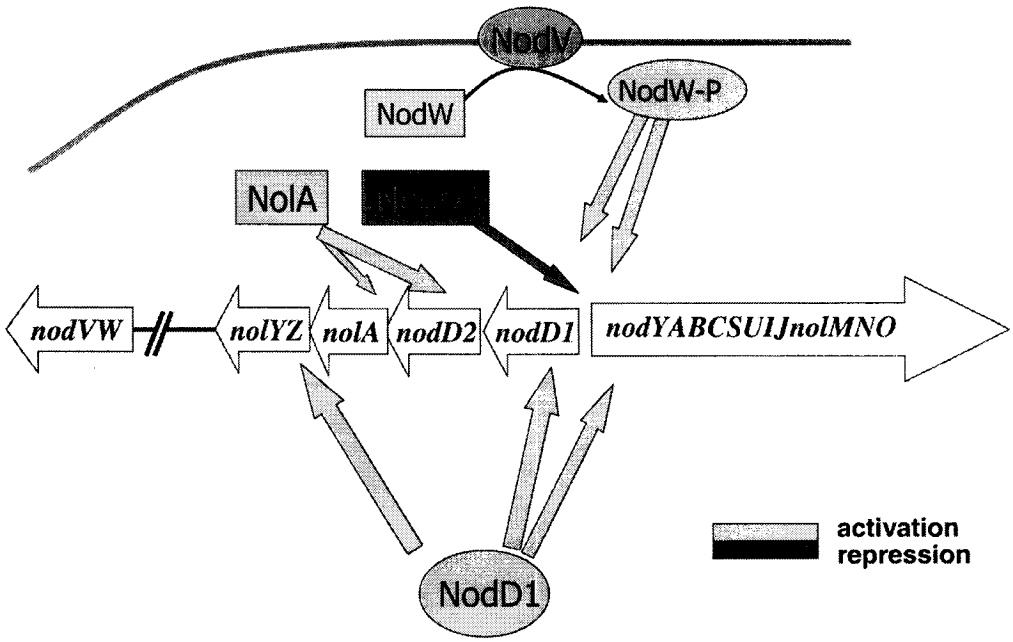
In addition to positive activation, the *nod* genes are also subject to negative regulation (Cren *et al.*, 1995, Fellay *et al.*, 1998, Garcia *et al.*, 1996, Kondorosi *et al.*, 1989). In *S. meliloti* repression is mediated by NolR, while in *Rhizobium sp.* strain NGR234 and *B. elkani* NodD2 acts as a repressor. Mutations to these repressors resulted in aberrant and delayed nodulation phenotypes (Fellay *et al.*, 1998, Kondorosi *et al.*, 1989). In *B. japonicum*, NolA and NodD2 form two key components in the negative regulation of the *nod* genes. Under appropriate conditions NolA induces NodD2 expression, which in turn represses the *nod* genes (Figure 2.2, 2.3).

Sadowsky *et al.* (1991) were the first to identify the NolA gene. It was classified as a genotype-specific gene since it was required for *B. japonicum* to nodulate restrictive soybean genotypes. NolA is a member of the MerR family of transcriptional regulators. The nolA gene has three functionally distinct proteins (i.e., NolA1, NolA2, and NolA3) (Loh *et al.*, 1999) (Fig. 2). NolA1 regulates the expression of both NolA2 and NolA3. However, the exact functions of both NolA2 and NolA3 are less clear. Strong expression of NolA2 appeared to reduce nodulation efficacy on soybean, while NolA3 was essential for nodulation of the same host (Loh *et al.*, 1999). However, the presence of multiple forms of NolA suggests that nolA plays a key role in allowing *B. japonicum* to nodulate its plant hosts.

In addition to regulating NolA2 and NolA3, NolA1 is also involved in controlling the expression of NodD2 which is a known repressor of the nodulation genes (Fellay *et al.*, 1998; Garcia *et al.*, 1996; Gillette and Elkan, 1996). Thus, NolA and NodD2 are the key components in regulating the negative expression of the *nod* genes. The importance of both these proteins is apparent in feedback regulation as well as quorum regulation of the *nod* genes.

Figure 2.2. Regulation of *Bradyrhizobium japonicum nod* genes.

Soybean plant roots exude the isoflavonoids genistein and diadzein. In response to these isoflavonoid signals, the *nod* genes are activated by NodD1 and NodVW, leading to the biosynthesis of Nod factors. Gene products of *NolA* and *NodD2* are involved in negative regulation of *nod* genes. NodD2 is regulated by NolA, which then suppresses the *nod* genes. (Adapted from Loh and Stacey, 2003)



d) Feedback regulation of the *nod* genes

Feedback regulation is a common control mechanism in bacteria. It allows bacteria to regulate the final product level and type of a biosynthetic operon (for examples see references Blasi and Bruni, 1981; Delorme *et al.,* 1999; Goldberger, 1974; Yanofsky *et al.,* 1981). Feedback regulation is usually mediated by the end products of the same operon, which reduces or suppresses the production of these products. Similarly *nod* genes of *B. japonicum* are also feedback regulated by the products of *nod* genes (Nod factors) or components of Nod factors (Loh and Stacey, 2001). Nod factors of *B. japonicum* contain either a tetrameric or pentameric chitin backbone (Cohn, *et al.,* 1999). Interestingly, only Nod factors containing a tetrameric backbone are able to feedback regulate the *nod* genes. Exogenous application of chitin tetramer also reduced the levels of *nod* gene expression. Chitin pentamer or other chitin oligomers (n = 1, 2, 3, 5, and 7) have little effect in this regard. Since *B. japonicum* produces both tetrameric and pentameric Nod factors (Cohn *et al.,* 1999), these findings suggest that *B. japonicum* senses only the levels of the chitotetraose molecules, which, when at or above a critical threshold, feed back regulates the *nod* operon. Increased expression of NolA and NodD2 occurs at high level of tetrameric Nod factors which then represses the *nod* operon.

Feed back regulation of *nod* genes is extremely important for effective and efficient nodulation. Strains with defective *nod* operon feed back regulation form abnormal nodules. Legume plants inoculated with strains with mutated nolA or nodD2 (Garcia *et al.,* 1996; Gillette and Elkan, 1996) show aberrant nodule phenotypes. On the other hand, strains that show over expression of Nod factors also resulted in delayed and aberrant nodule phenotype (Knight *et al.,* 1986).

e) Quorum regulation of the *nod* genes

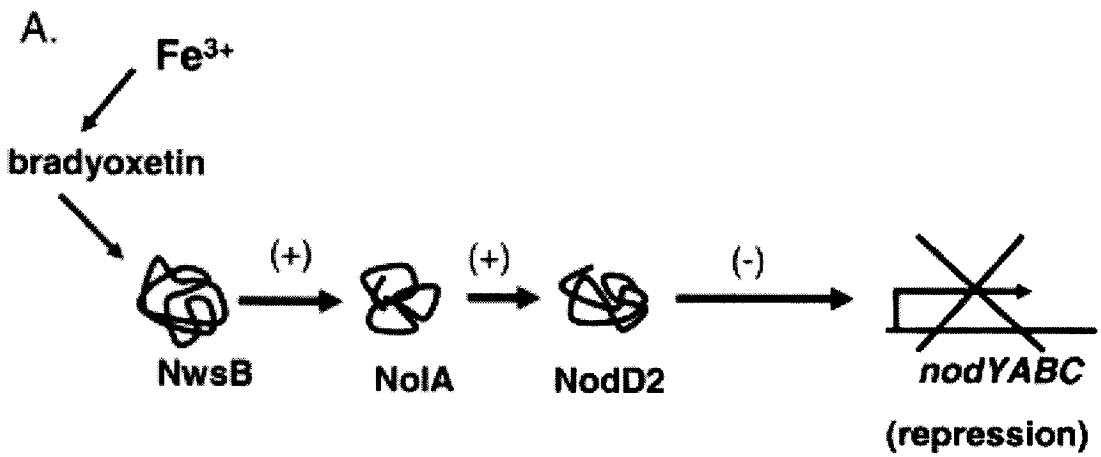
It has recently been shown that the *nod* genes are regulated in a population density-dependent manner (Loh *et al.,* 2001). This population dependence is similar to quorum sensing regulation found for gram-negative and gram-positive bacteria. Loh *et al.* (2001) have shown that the *nod* genes are maximally induced by the plant-to-bacteria signal,

genistein, at low population densities. Induction of *nod* genes is significantly reduced when bacterial cultures reach high cell population densities (Loh *et al.*, 2001). This quorum sensing is regulated by NolA and NodD2 (Loh *et al.*, 2001) (Figure 2.3). Both NolA and NodD2 accumulate with increasing population density. Strains with mutant *nolA* gene resulted in derepression of the *nodYABC* operon, even at high culture densities. Further studies have shown that this population density dependent expression of *nolA* is mediated by an extracellular signal molecule (termed CDF, for cell density factor) that accumulates in high-population cultures. This small molecule, initially described as CDF and shown to induce NolA expression, was later identified as bradyoxetin, [2-(4-{[4-(3-aminooxetan-2-yl) phenyl](imino) methyl}phenyl)oxetan-3-ylamine] (Loh *et al.*, 2002) (Figure 2.3).

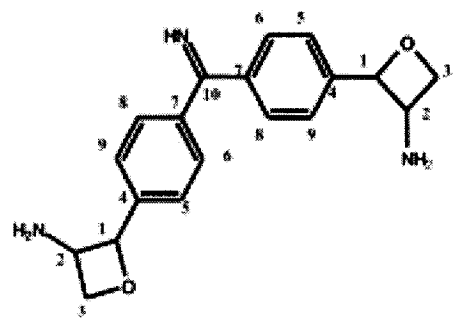
It is known that inside nodules, the *nod* genes are not expressed (Schlaman, *et al.*, 1991). The mechanism of this repression is also based upon quorum sensing. Inside nodules bacteria are packed in symbiosomes, resulting in a high cell-to-volume ratio, an environment of high population density which leads to quorum based repression of the *nod* genes. Loh *et al.* (2001), using a *nodY*-GUS fusion in strains with a mutated *nolA* gene, demonstrated that *nod* gene expression remained high in nodules while it was repressed in wild type nodules (not carrying *nolA* mutation). This demonstrates that *in-planta*, *nod* gene repression is mediated via NolA-mediated quorum sensing.

Figure 2.3. (A) Components involved in the population density-dependent regulation of the *nod* genes. In response to bradyoxetin, NwsB regulates the expression of *NolA* and *NodD2*. The expression of *NodD2* leads to the repression of the *nodYABC* operon in *B. japonicum*. In addition, expression of bradyoxetin is known to be regulated in response to iron. The addition of iron leads to reduced *NolA* expression and a concomitant increase in *nod* gene expression. **(B) Structure of bradyoxetin.**

(Adapted from Loh and Stacey, 2003)



B.



2.2.3. Bacteria-to-plant signal molecules: Nod factors (LCOs)

a) Biosynthesis and secretion of Nod factors

The nodulation and nitrogen fixation genes (*nod, nif* and *fix* genes) in *Rhizobium* species are located on large transmissible symbiotic plasmids (*Psym*) (Martinez *et al.,* 1990) while in *Bradyrhizobium,* these symbiotic genes are located on the chromosome (Long, 2001). These *nod* genes have been characterized as either 'common', such as *nod*ABCIJ, which show significant homology among *Rhizobium* and *Bradyrhizobium* species, or 'host-specific', such as *nod*Z of *B. japonicum, nod*EFLMN of *R. leguminosarum* and *nod*EFGHQ of *R. meliloti* (Horvath *et al.,* 1986; Stacey *et al.,* 1995). It is generally accepted that most of the *nod* genes are not expressed until the bacterial inducers of a compatible host plant are present.

The flavonoid inducible rhizobial *nod* genes have arbitrarily been named *nod, nol,* and *noe* genes (Spaink, 1995). These genes code for various proteins and their coordinated expression leads to the biosynthesis of LCOs. The common *nodABC* genes are responsible for the biosynthesis of the basic structure of Nod factors which is comprised of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) and an attached acyl group. The gene products of NodA, NodB and NodC are proteins that function as acyl transferase, chitin oligosaccharide deacetylase, and chitin oligosaccharide synthase, respectively, which play a central role in the biosynthesis of the basic LCO structure (Atkinson *et al.,* 1994; Geremia *et al.,* 1994; Spaink *et al.,* 1994). In addition to common *nodABC* genes most rhizobia also have host specific (*hsn*) nodulation genes (Kondorosi *et al.,* 1984). The activity of these Nod proteins provides different groups to the basic Nod factor structure which are believed to be responsible for host specific nodulation of legume plants (for review, see Carlson *et al.,* 1994).

In the soybean-*Bradyrhizobium* symbiosis, soybean roots exude isoflavonoid molecules (Parke *et al.,* 1985; Lameta and Jay, 1987; Rao and Cooper, 1994) which induce the coordinated expression of bacterial *nod* genes. These *nod* genes encode enzymes involved in the biosynthesis of Nod factors, which are structurally identified as lipo-chitooligosaccharides (LCOs) (Carlson *et al.,* 1993). It is well understood that

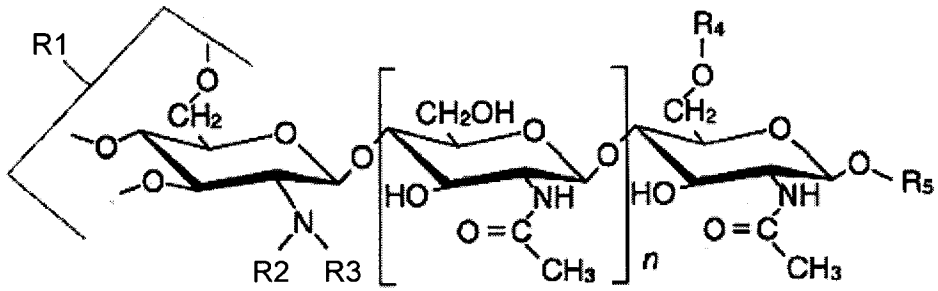
rhizobia produce a diverse group of LCOs. The diversity of LCOs is so such that even LCOs produced by a single bacterial cell are structurally different from each other. However, they all share a common backbone comprised of β-1,4-linked *N*-acetylglucosamines. It is speculated that host receptors at the tip of the root hair recognize the rhizobial LCOs and induce root hair deformation and root cortex cell division (Kosslak *et al.,* 1987; Mylona *et al.,* 1995; Prithiviraj *et al.,* 2000). A given rhizobial strain produces LCO molecules that have characteristic side groups (R_1-R_5) on both their non-reducing and reducing terminal glucosamine residues. These residues play a central role in determining the legume host range of a given bacterial strain. Research studies have shown that *Bradyrhizobium japonicum* strains produce a large variety of LCOs. Most of the *Bradyrhizobium japonicum* LCOs have 4-5 *N*-acetylglucosamines with different substitutions at both reducing non-reducing ends. The common and most active factor is a pentamer of N-acetylglucosamine acylated at the non-reducing end with vaccenic acid (18:1) and substituted at the reducing terminal sugar with 2-*O*-methylfucose (Sanjuan *et al.,* 1992; Carlson *et al.,* 1993; Stacey *et al.,*1995). LCOs are active at very low concentrations and nanomolar (10^{-9}M) concentrations can induce plant responses that lead to nodule organogenesis. Nod factor production at optimal concentrations contributes to efficient nodule formation.

Research studies have shown that the biosynthesis of LCOs occurs in the cytosol and inner membrane since the majority of Nod-enzymes are found in that region (Carlson *et al.,* 1995). Once biosynthesized, Nod factors can exist in two forms: free in the medium following bacterial secretion or as a constituent inserted into the bacterial membrane via its lipid moiety, with its glucosamine backbone extending into the extra-cellular medium (Hirsch, 1992). The products of *NodI* and *NodJ* facilitate the transportation of LCOs (Fernández-López *et al.,* 1996). NodI and NodJ proteins are found in the inner membrane and it is suggested that they may be involved in the translocation of LCOs from the cytoplasm to the periplasm (Schlaman *et al.,* 1990). It is thought that NodT in conjunction with NodI and NodJ form a secretion system that exports the LCOs directly into the medium (Spaink, 1995).

b) Nod factor structure

Rhizobia produce a diverse group of LCOs which vary from species to species. This diversity is so widespread that even a single strain produces several different LCOs. The variation in structure of different LCOs is due to: i) the number of GlcNAc units, where most LCOs have 3 to 5 GlcNAc units in length, ii) the structure of attached fatty acyl moieties, and iii) the strain-specific substitutions e.g carbamoyl, acetyl, 2-O-methyl fucose, etc. groups (Spaink, 1996). *Bradyrhizobium japonicum* produces structurally diverse LCOs, however all strains produce a common Nod factor NodBj-V (C18:1, MeFuc); that is a chitose pentasaccharide substituted at the reducing end with 2-O-methylfucose and acylated with a 18:1 fatty acid at the non-reducing end. Generally, *B. japonicum* strains produce Nod factors that carry 4 to 5 *N*-acetyl-D-glucosamine (GlcNAc) residues attached to different groups like 2-O-methylfucose, Fuc, Acetyl, Carbamyl etc. The fatty acyl chain is generally 16 or 18 carbons long with or without saturation (for review, see Stacey *et al.,* 1995) (Figure 2.4).

Figure 2.4. Nod factors produced by *B. japonicum*. Nod factors of *B. japonicum* have 4-5 *N*-acetylglucosamine units, and this basic structure is common to all Nod factors produced by *B. japonicum.* The "n" represents the number *N*-acetylglucosamine units. The basic structure can have different substitutions denoted at R1, R2, R3, R4 and R5. In the Nod metabolite NodBj-V($C_{18:1}$,MeFuc), the "Nod Bj' means that the Nod factor is produced by *Bradyrhizobium japonicum,* "V" means 5 *N*-acetylglucosamine units, 'C18:1' means a fatty acyl group with 18 carbons with one double bond (vaccinic acid), and the 'MeFuc' means a methyl fucose group is attached to the basic structure (Stacey *et al.,* 1995).



Nod metabolite	Strain	R1	R2	R3	R4	R5	n	$(M+H)^+$
NodBj-V($C_{18:1}$,MeFuc)	110 135 61	H	$C_{18:1}$	H	2-O-MeFuc	H	3	1416
NodBj-V(Ac,$C_{18:1}$,MeFuc)	135 61	Ac	$C_{18:1}$	H	2-O-MeFuc	H	3	1458
NodBj-V($C_{16:0}$,MeFuc)	135	H	$C_{16:0}$	H	2-O-MeFuc	H	3	1390
NodBj-V(Ac,$C_{16:0}$,MeFuc)	135	Ac	$C_{16:0}$	H	2-O-MeFuc	H	3	1432
NodBj-V($C_{16:1}$,MeFuc)	135	H	$C_{16:1}$	H	2-O-MeFuc	H	3	1388
NodBj-V(Cb,$C_{18:1}$,NMe,MeFuc)	61	Cb	$C_{18:1}$	Me	2-O-MeFuc	H	3	1473
NodBj-V(Ac,Cb,$C_{18:1}$,MeFuc)	61	Ac,Cb	$C_{18:1}$	H	2-O-MeFuc	H	3	1501
NodBj-IV($C_{18:1}$,MeFuc)	61	H	$C_{18:1}$	H	2-O-MeFuc	H	2	1213
NodBj-IV(Cb,$C_{18:1}$,MeFuc)	61	Cb	$C_{18:1}$	H	2-O-MeFuc	H	2	1256
NodBj-IV($C_{18:1}$,Fuc,Gro)	61	H	$C_{18:1}$	H	Fuc	Gro	2	1273
NodBj-IV($C_{18:1}$,NMe,Fuc,Gro)	61	H	$C_{18:1}$	Me	Fuc	Gro	2	1287
NodBj-IV(Cb,$C_{18:1}$,Fuc,Gro)	61	Cb	$C_{18:1}$	H	Fuc	Gro	2	1316
NodBj-IV(Cb,$C_{18:1}$,NMe,Fuc,Gro)	61	Cb	$C_{18:1}$	Me	Fuc	Gro	2	1330

2.2.4. Nodule formation

a) Inter-organismal signal exchange

It is suggested that Nod factor receptor(s) present on the tip of the root hairs is essential for the signal transduction cascade leading to the early events of nodulation such as root hair curling, bulging, and wiggling. In soybean the Nod signal receptor is proposed to be a member of a general class of chitin-binding proteins (Stacey, 1999). This putative Nod factor-receptor binding reaction is presumed necessary for bacterial signal perception by the host plant (Diaz *et al.,* 1989). The N-glucosamine residues of the Nod factor react with a sugar-binding site of the receptor. The strength of the interaction between Nod factor(s) and the receptor depends on several factors, such as the length of the glucosamine backbone, the presence or absence of various side substitutions and the composition of the lipid side chain (Hirsch, 1992). It has also been suggested that Nod factors are involved in the inhibition of salicylic acid-mediated defense mechanisms in legumes; this could explain why rhizobia can successfully infect legume plants without triggering a host defense response (Pinton *et al.,* 2001). Besides the flavonoid and the LCO that are exchanged between legume plants and rhizobial cells, it is now believed that a multitude of additional signals from both symbiotic parties are likely involved in the nodulation process. These additional signal molecules include bacterial surface polysaccharides and excreted proteins (Stacey, 1999). In the interactions between soybean and *B. japonicum*, the bacterial cyclic β-glucans may play a role in suppressing the host defense responses (Perret *et al.,* 2000).

b) Bacterial Attachment and Root hair Response

Once compatible signaling between the two symbionts is established through the Nod factor-receptor reaction, the bacteria attach to the root hair tip, first as loose binding of the bacteria to the root hair surface and anchoring of the bacteria to the surface of the root hair, leading to a firm attachment, called 'cap formation'. It is believed that first process of interaction is mediated by calcium binding protein, rhicadhesin while the second step is

mediated by plant lectins and bacterial appendages, such as cellulose fimbriae and rhizobial lectins, in the case of *B. japonicum.* Although there are probably several factors mediating the bacterial attachment yet to be discovered, the binding of Nod factor already existing in the cell membrane with a host plant lectin is the most apparent selective mechanism (Vlassak and Vanderleyden, 1997). Root hair tip attachment positions the bacteria at a viable invasion site.

After initial attachment and perception by the plant root hairs, the root hair is transformed into an extending tubule that elongates by tip growth. The root hair tip has a high level of physiological activity and a thin, unpolymerized cell wall. During the attachment stage, the root hair may supply carbon and nitrogen nutrients for the bacteria by means of exudates (Peterson and Farquhar, 1996). The initial events involved in the signal exchange and recognition of bacteria also lead to morphological changes in the root hair: root hair deformation (Had), including swelling, twining, branching and other forms of deformation, and root hair curling (Hac) (Higashi and Abe, 1980; Callaham and Torrey, 1981). Experiments with purified LCO demonstrated that LCO itself is responsible for inducing these root hair responses (Kijne, 1992; Prithiviraj *et al.,* 2000). In general, the root hair deformation marks the first visible event in the nodulation process. The protein hadulin may be involved in the root hair deformation or curling and is one of the root hair-specific proteins induced by the Nod factor(s) (Krause and Broughton, 1992). In the soybean-*B. japonicum* symbiosis, bacterial attachment to root hairs occurs within minutes after inoculation, while marked root hair curling occurs within 12 hours (Turgeon and Bauer, 1982; Prithiviraj *et al.,* 2000). The infectible cells on soybean roots appear to be located just above the zone of root elongation and just below the position of the smallest emergent root hairs (Bhuvaneswari *et al.,* 1980). In general, only short root hairs, which have just emerged at the time of inoculation, become tightly curled. Markedly curled root hairs occurred in patches. Longer root hairs do not exhibit pronounced curling although they are often deformed (Turgeon and Bauer, 1982).

c) Infection Thread and Symbiosome

Infection thread formation is believed to result from bacterial polysaccharide-degrading enzymes that hydrolyze the plant cell wall in the curled region. This process leads to invagination of the plasma membrane and new plant cell wall material is deposited (Kijne, 1992; Mateos *et al.,* 2001). As a result, the formation of a tubular structure, called the infection thread, is formed. Thus, the infection thread is a continuation of the plant cell wall. The ultrastructure of the infection thread wall is very similar to the normal plant cell wall, but the incorporation of certain nodulins may endow it with unique properties. Both plant nodulins and hadulin are involved and expressed during infection thread formation (Scheres *et al.,* 1990). Infection threads in soybean are relatively narrow, with a diameter of about 1 μm, and contain a single row of linearly aligned bacteria (Turgen and Bauer, 1985). The bacteria in the infection thread are surrounded by a matrix consisting of compounds secreted by both the plant and the bacteria (Rae *et al.,* 1992).

During infection thread growth, the root hair nucleus precedes the infection thread tip. The bacteria travel via the infection thread, following the nucleus. Microtubules might be responsible for anchoring the nucleus to the root hair tip. Concomitant with infection thread formation, cortical cells are mitotically reactivated, forming the nodule primordium. The infection thread grows toward this primordium and upon reaching there, releases bacteria into the cytoplasm that are still surrounded by plant membrane (Kijne, 1992; Goodchild and Bergersen, 1966). Under greenhouse conditions, soybean infection threads can be observed 24 h after inoculation with *B. japonicum*. By 48 hours, the infection threads have progressed to the bases of the curled root hairs but have not penetrated into the cortex cells (Turgeon and Bauer, 1982; Zhang and Smith, 1995). In seed-inoculation experiments, electron microscopy photographs show that 10–12 days after inoculation and planting, *B. japonicum* cells are freed from the infection thread by disintegration of the infection thread wall. The disintegrated wall materials are enclosed in membrane-bound vesicles. As the thread wall is removed in this way, the bacteria are each enclosed in an envelope derived from the thread membrane and then released into the host cytoplasm. The residual thread wall material remaining around a bacterium is removed from the envelope space by vesiculation of the membrane envelope. Both the

bacteria and the infection thread wall material enter the host cell by endocytosis (Bassett, 1977). Each bacterial cell surrounded by the envelope forms an organelle-like structure termed as symbiosome. In soybean, a symbiosome consists of a host-derived membrane, the symbiosome membrane, which envelops a microsymbiont and creates an internal space called the symbiosome space. The symbiosome membrane is generally referred to as the peribacteroid membrane (PBM). The symbiosomes continue to divide, with the bacteria and PBM dividing in concert, to increase the bacterial population in the infected host cells (Day, 1999).

d) Nodule ontogenesis

When the infection thread approaches root cortical cells, these cells begin to divide, beginning either in the outer or inner cortex of the root, depending on nodule type. The nodules that develop are either indeterminate or determinate. The indeterminate nodules are elongate and club-shaped for example clover, alfalfa, vetch and pea. The determinate nodules are spherical, for example those formed on soybean, mungbean, and common bean. In determinate nodules the initial cortex cell division occurs in the outer cell layers and lacks a meristem. Cell division ceases early during nodule development and the final form of the nodule results from cell enlargement rather than cell division (reviewed by Hirsch, 1992).

Determinate nodules can be broadly subdivided into central and peripheral tissues. The peripheral tissues consist of nodule parenchyma and nodule cortex separated from each other by the nodule endodermis. Vascular bundles are embedded in the nodule parenchyma. The nodule cortex, at the outside of nodule endodermis, is derived from the root cortical cells that surround the nodule primordium. In the central tissue of the nodule, only some cells contain bacteria. The infected cells are larger and denser due to the presence of released bacteria. Interspersed among the infected cells are small, highly vacuolated, uninfected cells. In soybean these interstitial cells, which contain a nodule specific uricase, outnumber the infected cells by a ratio of approximately 3:2 (Vandenbosch and Newcomb, 1986; reviewed by Hirsch, 1992).

With the developing nodules, bacterial cells also change in conformation. At this stage, bacterial cells inside the symbiosomes differentiate into nitrogen fixing bacteroids and stop dividing. In determinant nodules, the peribacteroid membrane (PBM) of adjacent symbiosomes appears to fuse, forming multiple bacteroid symbiosomes (Day, 1999). The bacteroids are now able to fix atmospheric dinitrogen. *Bradyrhizobium* bacteroids contain three respiratory chains that are different from the free-living cells. In the bacteroid, poly-β-hydroxybutyrate (PHB) is accumulated and dinitrogenase is expressed at up to 10 % of the total soluble protein (reviewed by Werner, 1992). Mature infected cells of soybean nodules contain many thousands of multiple bacteroid symbiosomes actively fixing nitrogen (Day, 1999).

2.2.5. Soybean Nodule Function

a) Carbon Metabolism

The process of nitrogen fixation requires a large input of energy which is provided by the plant. In most rhizobium/legume symbiosis, plants provide photosynthates in the form dicarboxylic acid such as succinate, fumarate and malate (Driscoll *et al.,* 1995). The translocation of photosynthate, mainly sucrose, occurs via phloem and this is broken down by sucrose synthase to glucose and fructose. Some glucose is oxidized via the Embden-Meyerhof pathway and the tricarboxylic acid (TCA) cycle in host cells to produce malate and succinate. The peribacteroid membrane is relatively impermeable to sugars but does contain a dicarboxylate transporter capable of rapidly transferring C_4-dicarboxylic acids to the bacteroid. Bacteroids are capable of taking up C_4-dicarboxylates at rates significantly higher than that of sugars such as glucose (reviewed by Mitsch *et al.,* 1999). C_4-dicarboxylates in bacteroids are oxidized dominantly via the TCA cycle producing energy and some intermediates. To maintain the TCA cycle with only C_4-dicarboxylates as the sole carbon source, a pathway to generate acetyl-CoA is essential. Acetyl-CoA is readily produced from pyruvate under the catalysis of pyruvate dehydrogenase. Although several enzymatic pathways exist in rhizobial cells, malic enzymes are the dominant means for production of pyruvate from malate in bacteroids

(Mitsch *et al.,* 1999). Besides supplying C4-dicarboxylic acids to bacteroids, in the infected and uninfected nodule cells, a significant proportion of the glucose-6-phosphate is used to form starch, stored in the amyloplasts. Nitrogen fixation is a high energy process and it is estimated that the cost of fixing one gram N in a soybean nodule is 12.1 gram of carbohydrate. Of this 60 % is spent in N_2 fixation and H_2 evolution (reviewed by Werner, 1992).

b) Oxygen Protection and Supply

The nitrogenase enzyme complex consists of two enzymes: dinitrogen reductase and dinitrogenase. Dinitrogenase reductase is irreversibly inactivated by oxygen. As a result nitrogen fixation cannot be carried out in the presence of oxygen. For this reason, in legume nodules the average O_2 concentration is very low. However, bacteroids need oxygen for respiration and hence there should be tight control of oxygen supply to the nodule tissue. The legume nodules exercise physiological control over the O_2 concentrations in their infected cells by adjusting their permeability to O_2 diffusion. Generally speaking, the nodules have a two-stage mechanism to control the O_2 diffusion: coarse control by the nodule inner cortical barrier and fine control through an innate, homeostatic control in the infected cells (Layzell, 1999). The nodule parenchyma has very few and small intercellular spaces. Most of the O_2 and other gases must enter the nodule by diffusing through the intercellular spaces. Oxygen diffusion is about 10^4 times faster through air than through water. The plant has the capacity to allow these spaces to fill with water or to keep them partially or maximally emptied of water. The concentration of some organic and inorganic components in the water-filled diffusion barrier is also a part of the diffusion resistance. The potassium concentration in effective and ineffective nodules of soybean is significantly different. In the nodule parenchyma, some nodulin genes such as GmENOD2 and GmENOD13 are expressed, and these protein products might contribute to the development of the oxygen diffusion barrier (Mylona *et al.,* 1995; Hirsch, 1992). The plant cells within the nodule produce leghemoglobin, a protein which serves as an oxygen carrier to the bacteroids within the nodule. This enables the bacteroids to obtain enough oxygen for respiration but ensures

that the oxygen is in a bound form so that it cannot harm nitrogen fixing enzymes inside the bacteria. Cutting open a nodule reveals the deep red color typical of leghemoglobin when it holds bound oxygen. The leghemoglobin concentration is very high in the infected cells of nodule central tissue. Leghemoglobin, as a late nodulin constituting up to 20% of the total soluble nodule protein, plays a large role in the nodule O_2 concentration control (Werner, 1992).

c) Nitrogen Fixation

Nitrogen fixation in legume nodules follows the general equation:

$$N_2 + 8e^- + 8H^+ + 16\text{Mg-ATP} \rightarrow 2NH_3 + H_2 + 16\text{Mg-ADP} + 16\ \text{Pi}$$

The nitrogenase complex is made up of two fairly different enzymes, dinitrogenase and dinitrogenase reductase. Dinitrogenase, the larger one, contains Mo, Fe and Co and consists of 4 subunits ($\alpha_2\beta_2$) with a total molecular weight of 200 to 240 KD. It is termed as MoFe protein (Yates, 1992). Its Mo atom exists in a cofactor with configuration of 7 Fe, 8 S and 1 Mo called FeMoco (Yates, 1992). This cofactor is the site of N_2 binding and reduction. Dinitrogenase reductase, the smaller enzyme, contains 4 Fe atoms and 4 acid labile sulphurs. It is termed the Fe protein and is made up of two identical subunits (α_2) with a total molecular weight of 55 – 65KD (Yates, 1992). For the reduction of the N_2 bound in FeMoco, the 8 electrons are transferred from the Fe protein to the MoFe protein in a MgATP-dependent way. Both the Fe protein and the MoFe protein are oxygen sensitive. In the absence of an added reducible substrate nitrogenase reduces protons (H^+) to H_2 (reviewed by Yates, 1992). The produced H_2 may be partially or entirely recycled by bacterial strains with uptake hydrogenase (Hup^+). Strains that are Hup^- are not able to recycle H_2, and in these cases H_2 evolution can be measured to monitor nitrogenase activity (Hunt and Layzell, 1993).

In eastern Canada field grown soybean can fix 100-200 kg N/ha/yr (Smith and Hume, 1987). Soybean gets 25 – 75 % of the total N needed by fixing atmospheric N_2 (Deibert et al. 1979). Seed yield in nodulated soybean plants is generally comparable to plants supplied with N fertilizer (Yates, 1992).

d) Ammonia Assimilation

In legume nodules the majority of N_2 reduced by the bacteroids is transferred to the plant. It is incorporated into organic compounds through the ammonium-assimilation pathway, which operates in free-living bacteria, but can be repressed in the symbiotic bacteroids (Rosendahl *et al.,* 1999). Most of the fixed nitrogen exists in the form of ammonium (NH_4^+) under the physiological conditions inside the bacteroids. Ammonium is initially incorporated into alanine in the bacteriods, then transported into the host cells through the inner and outer membrane of the bacteroid cells and through the peribacteroid membrane of the symbiosome (Waters *et al.,*1998). In soybean nodules, the alanine is synthesized from pyruvate and ammonium by L-alanine dehydrogenase. *B. japonicum* alanine dehydrogenase is sufficiently rapid that the ability of bacteroids to assimilate ammonium into alanine is limited by the availability of ammonium produced by nitrogenase. Alanine is most likely only a transport molecule as glutamate is the central compound of the nitrogen metabolism in most organisms. The use of alanine as a transport molecule would functionally and spatially distinguish bacteroid nitrogen metabolism and transport from plant host cellular nitrogen metabolism. This would facilitate the rapid movement of fixed nitrogen from the bacteroid to plant (Allaway *et al.,* 1999; Emerich *et al.,* 1999). In the host cell, ammonium is further assimilated through catalysis of the enzymes glutamine synthetase, glutamate synthase, asparate aminotransferase and asparagine synthetase. The overall reaction is:

$$2NH_3 + 3ATP + \text{oxaloacetate} + NADH + H^+ \rightarrow$$
$$\text{L-asparagine} + AMP + 2\ ADP + 2\ pi + NAD^+ + PPi$$

Soybean is a ureide-exporting legume. Ammonium in the host cells is used for the amination of specific organic acids to produce amino acids, such as glutamate, aspartate and glycine. The glutamine, glycine and aspartate are then used for purine synthesis in plastids of infected cells. In the neighboring uninfected cells, purine nucleotides are transformed to xanthine and uric acid, which is oxidized to produce allantoin. Allantoin is further transformed to allantoic acid in the smooth endoplasmic reticulum (ER) of uninfected cells. Uricase II, a nodulin located in the peroxysomes of the

uninfected cells, is involved in production of ureides which are exported to the plants via xylem (reviewed by Werner, 1992).

2.3. Jasmonates as signal molecules

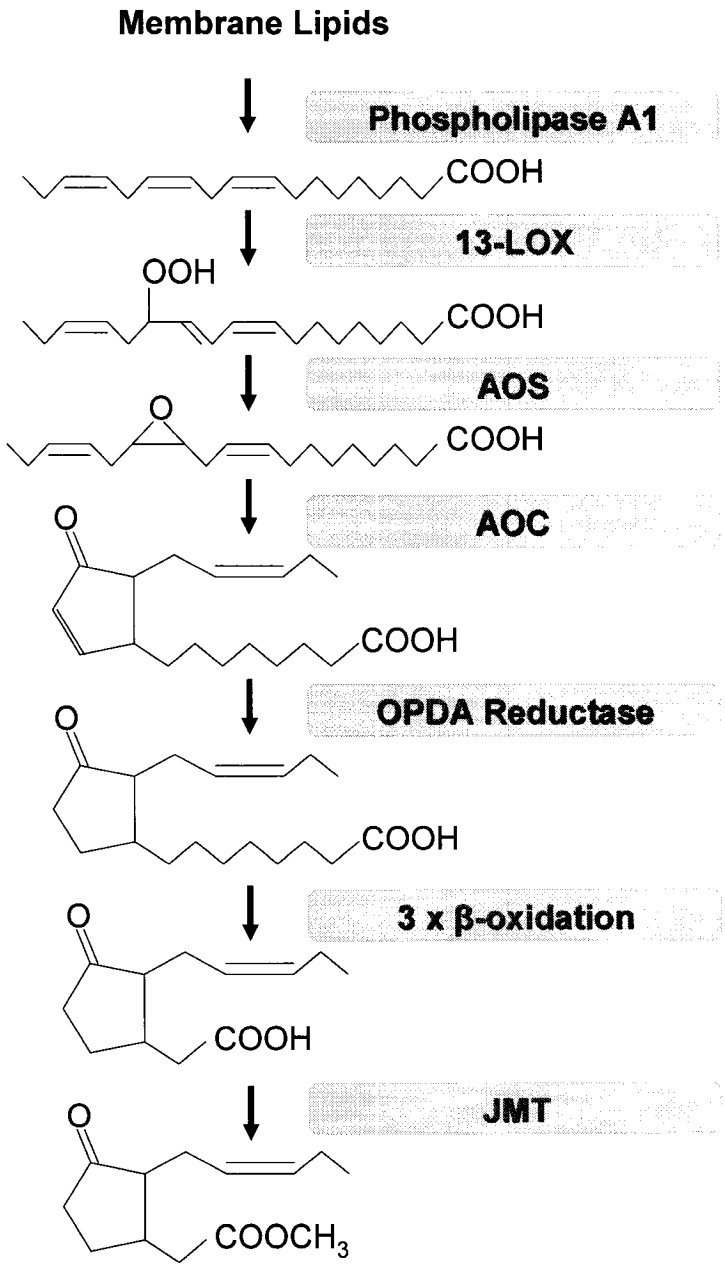
Jasmonates, such as jasmonic acid and its methylated derivative, methyl jasmonate, are cyclopentanone derivatives, naturally occurring growth regulators found in higher plants (Meyer *et al.,* 1984). JA is biosynthesized from α-linolenic acid, a C18 poly-unsaturated fatty acid, via the octadecanoid pathway (Figure 2.5). It is thought that linolenic acid is produced from membrane lipid breakdown by the action of phospholipase. These fatty acids are converted into 13-hydroperoxylinolenic acid, which is converted into 12, 13 epoxy-octadecatrienoic acid, which is then catalyzed into 12-oxo-phytodienoic acid (12-OPDA). Through reduction and three ß-oxidation steps, 12-OPDA is converted to (+)-7-iso-JA (Creelman and Mullet, 1997, Vick and Zimmerman, 1984) (See Figure 2.5). The key enzymes involved in the octadecanoid pathway include lipoxygenase (LOX), which adds oxygen to the fatty acid, allene oxide synthase (AOS), and allene oxide cyclase (AOC). The term jasmonates includes cylcopentenones (eg. OPDA) and cyclopentanones like JA and MeJA.

Jasmonates are ubiquitous in plants and have several well known roles in plant growth and development. They play an active role in leaf abscission, and senescence; inhibit plant growth and seed germination (Corbineau *et al.,* 1988) and are involved in induced disease resistance (Gundlach *et al.,* 1992). The central role of JA in wounding and osmotic stress induced signal transduction is well investigated (Kramell *et al.*, 1995).

One remarkable feature of jasmonates is that some of its members act as signal molecules outside of plants and serve alternative functions. For instance, the volatile member of jasmonate pathway *cis*-jasmone (cJ) or (*Z*)-jasmone is released from plant leaves due to damage or insect herbivory and may help repel herbivorous species and attract their predators (Birkett *et al.,* 2000). Besides plant-insect communication, Methyl jasmonate (MeJA), a volatile derivative of JA, acts as a signal in inter-plant communication that can occur from leaves of one species of plant to leaves of another species to activate the expression of defensive genes (Farmer and Ryan, 1990).

Besides their *ex-planta* roles in plant-insect communication and inter-plant communication, the presence of jasmonates has been reported in the rhizosphere (Pareek and Guar, 1973). The presence of JA in the rhizosphere is due to its rhizosecretion from plant roots. Dathe *et al.* (1994) reported that the exudation of JA from wheat seedling roots is much higher than its concentration in the roots. Germinating soybean seeds also exude large quantities of JA (Creelman and Mullet, 1995); seed germination and radicle emergence are critical stages for the establishment of the nitrogen fixing symbiosis. Despite their presence in the rhizosphere, there is little knowledge as to how jasmonates affect rhizosphere microorganisms, including rhizobia. One report has indicated an effect of jasmonates on the rhizobia; Rosas *et al.* (1998) reported that jasmonates can act as signaling molecules and can induce the expression of *nod* genes of *Rhizobium meliloti*. However, there is no detailed study of this class of compounds on the *nod* gene expression activity and Nod factor production of *Bradyrhizobium japonicum*. Thus, determination of the possible role of this class of molecule in the *Rhizobium*-legume symbiosis is an area potentially of great interest.

Figure 2.5. The biosynthesis of jasmonates in plants. The biosynthesis of jasmonates begins with fatty acid peroxidation of linolenic acid, which is believed to be produced from the plasma membrane via the action of phospholipase A1. The pathway is generally referred to as the octadecanoid pathway due to its 18 carbon precursor, linolenic acid. The names of the enzymes involved in this pathway are given in boxes. Key enzymes of the octadecanoid pathway are 13-LOX (13 lipoxygenase), AOS (Allene Oxide Synthase), AOC (Allene Oxide Cyclase), OPDA (12-Oxophytodienoid Acid) reductase, and JMT (Jasmonic acid Methyl Transferase) (Adapted from Cheong and Choi, 2003)


Membrane Lipids
Phospholipase A1
COOH
13-LOX
OOH
COOH
AOS
O
COOH
AOC
O
COOH
OPDA Reductase
O
COOH
3 x β-oxidation
O
COOH
JMT
O
COOCH3

2.4. Biological nitrogen fixation in soybean at low root zone temperatures (RZTs)

Nitrogen fixation, whether biological or industrial, is of primary importance for introduction of fixed N into the biosphere. The total N fixed during these two processes is estimated to be in the range of 100-290 million tonnes of N per year (Cleveland *et al.*, 1999). Estimated biological N fixation by agricultural crops is in the range of 40-48 million tonnes per year (Gallo-way *et al.*, 1995; Jenkinson, 2001) while industrial N fixation, applied to the crops in the form of fertilizers, accounts for up to 83 million tonnes of N per year (Jenkinson, 2001). Biological nitrogen fixation (BNF) is environmentally friendly and sustainable, as it is driven by solar energy trapped by crop plants through photosynthesis (Bohlool *et al.*, 1992).

Many factors influence legume nodulation and nitrogen fixation. Of the abiotic factors, combined nitrogen, pH, temperature, water status, salinity and soil texture are the most widely studied. Biotic factors influencing legume nodulation and nitrogen fixation are the rhizobial population density in the rhizosphere, the presence of other rhizosphere microbes and the interactions between the plant and the various rhizobacteria, as well as between the plants and the rhizobia themselves (Vlassak and Vanderleyden, 1997; Zahran, 1999). There have been several recent reviews of environmental factors influencing BNF (Broughton *et al.*, 2003; Dilworth *et al.*, 2001; Graham, 1992; Hungria and Vargas, 2000; Perret *et al.*, 2000; Serraj *et al.*, 1999; Sessitsch *et al.*, 2002; Walsh, 1995; Zahran, 1997 and 1991). We will restrict our discussion to the effect of low soil temperature on nodulation and nitrogen fixation and strategies to improve nodulation and nitrogen fixation under such conditions.

For optimal nodulation and nitrogen fixation, legume crops need an optimum temperature regime, which varies from species to species. When other factors are not limiting, legume crops have the ability to fix atmospheric N_2 in association with compatible rhizobia. Both high and low temperatures seriously affect all stages of nodulation and nitrogen fixation. Lowering root zone temperature (RZT) adversely affects nodulation and nitrogen fixation, thus prolonging the nitrogen starvation period that occurs between the time when cotyledonary reserves are exhausted and the onset of N_2 fixation. Shortening this period leads to increased crop growth and yield. For example,

soybean is a subtropical legume and its optimum temperatures are in the range of 25 to 30°C for nodule activity and growth (Jones and Tisdale, 1921). Soybean plant growth, nodulation and nitrogen fixation are inhibited when under of sub-optimal RZTs (below 25°C), and this ultimately affects total dry matter production and grain yield (Summerfield and Wien, 1982). Sub-optimal RZT restricts and delays nodulation and nodule function (Jones and Tisdale, 1921; Layzell *et al.*, 1984; Lynch and Smith, 1994) and this delay is temperature dependent. For instance, in soybean the time between inoculation and the onset of nitrogen fixation increases 2-3 days per °C as RZT drops from 25°C to 17°C, and about 7 days per °C when RZT declines below 17°C (Zhang and Smith, 1994; Zhang *et al.*, 1995). Low RZTs also reduce the N_2 fixation activity of the nitrogenase enzyme complex (Layzell *et al.*, 1984). Low RZT has also been shown to adversely affect nodulation and nitrogen fixation in common bean. Low RZT adversely affects nodulation and nitrogen fixation in common bean, pea and lentils. Low RZT delays the onset of nodulation and subsequent nodule development of legume crops. In common bean, the time between inoculation and nitrogen fixation is prolonged by 7 days when the root zone temperature drops from 25 to 17°C. This delayed nodulation and the onset of nitrogen fixation results in reduced amounts of fixed nitrogen and ultimately reduced plant growth (Poustini *et al.*, 2004). When pea plants were subjected to RZT treatments (10, 15, and 20°C), decrease in nodulation and nitrogen fixation, and slower plant development were observed. This phenomenon was temperature dependent (Lira Junior *et al.*, 2003). Besides, low RZT also inhibits nodulation and nitrogen fixation of lentils (Begum *et al.*, 2001b).

Studies with soybean subjected to low RZT have shown that the early infection, nodule initiation and further nodule development are strongly affected by low RZTs (Lynch and Smith, 1993; Zhang and Smith, 1994), which ultimately influences the time to the onset of nitrogen fixation. The delay of early infection processes in the *Bradyrhizobium*-soybean symbiosis is due to the low temperature induced disruption of the inter-organismal signal exchange, which occurs at the beginning of the establishment of symbiosis (Zhang and Smith, 1995). Indeed, low temperature decreases both the biosynthesis and rhizosecretion of genistein from soybean roots into the rhizosphere; genistein is an important signal molecule that induces the transcription of *B. japonicum*

nod genes (Zhang and Smith, 1996a). At the same time, low temperatures inhibit the transcription of *B. japonicum nod* genes (Zhang *et al.*, 1996). As indicated above, the product of the *nod* genes are Nod factors, identified as lipo-chitooligosaccharides (LCOs) (Carlson *et al.*, 1993). Nod factors are able to induce many of the early events in nodule formation (Stacey, 1992). The production and particularly secretion of the Nod metabolites are severely restricted as the temperature declines (McKay and Djordjevic, 1993). Recently, Zhang *et al.* (2002) have also shown that the ability of *B. japonicum* to produce Nod factors decreases at suboptimal growth temperatures. This demonstrates that low temperatures negatively affect both aspects of inter-organismal signal exchange, plant-to-bacteria and bacteria-to-plant, which is crucial for nodule initiation.

Besides detrimental effects of low RZT on the early signal exchange processes between the two symbionts, it also inhibits the function of nitrogen fixing nodules already established, and this inhibition may be associated with nodule oxygen permeability (Walsh and Layzell, 1986).

2.5. Agricultural uses of *nod* gene inducer molecules

The *nod* gene inducer molecules have tremendous potential in legume agricultural production systems, especially when the soil temperatures are inhibitory to legume nodulation and development. At low soil temperatures, nodule initiation, development, and nitrogen fixation are markedly reduced, resulting a prolonged period of N limited growth at the late seedling stage. This eventually leads to delayed crop maturity and lower yields. This is particularly important in Eastern Canadian soils where early vegetative growth of soybean occurs at soil temperatures below 17°C (10-cm depth) during the first two months of the growing season. Zhang and Smith (1994) found that the first 12 h post-inoculation were the most sensitive to low RZT. This is the crucial time in the process of nodulation since it is when the initial signal exchange between the two organisms takes place. This low RZT induced inhibitory effect on inter-organismal signal exchange can be partially overcome when plants are inoculated with bacteria pre-induced with genistein – a plant-to-bacteria signal molecule. This accelerated the early stages of nodulation leading to more rapid nodule development and earlier onset of nitrogen fixation (Zhang and Smith, 1994). Furthermore, genistein applied to seeds in the furrow at

the time of planting, accelerated nodulation and increased N_2 fixation at low soil temperatures, which would normally delay or inhibit nodulation (Zhang and Smith, 1996b, 1997). Pre-incubation with genistein also encouraged nodule occupancy by the inoculant (Pan and Smith, 2000). Similar findings have been reported for other legume crops. For instance, when pea and lentils were inoculated with hesperatin induced *Rhizobium leguminosarum* bv. viceae, nodulation and plant growth were increased under greenhouse and field conditions (Begum *et al.,* 2001a; Begum *et al.,* 2001b). The role of naringenin induced *R. leguminosarum* bv. viceae in promoting pea growth and development has also been established (Bandyopadhyay, 1996).

The technology of genistein as a signal molecule to enhance nodulation and nitrogen fixation by soybean has been present in the market place for several years. The commercial product 'SoyaSignal', which uses genistein and diadzein has been marketed in Northern America since 1997 (Smith and Zhang, 1996) and is applied in different ways to promote soybean nodulation and nitrogen fixation. 'Soysignal' can be applied either directly to the seed before sowing or in furrows in soils where adequate native populations of *Bradyrhizobium* are present. Research data collected over 6 years on 'Soyasignal' technology, from more than 100 field trials in Northern America, revealed that this technology significantly improves soybean nodulation and nitrogen fixation. On average, there was 7% increase in grain yield. However, the results of 'Soyasignal' application were temperature dependent and the strongest effects on yield were seen when applied to soils with cool springs with temperatures, lower than 17°C (Leibovitch, *et al.,* 2001). Although genistein based 'Soysignal' technology shows promise in enhancing soybean yield, the genistein it is both exceedingly expensive (even when purchased in industrial quantities they are on the order of \$100 g^{-1}) and harmful to the rhizobia being cultured for inclusion in legume inoculants, posing problems for product formulation.

2.6. Conclusions

Soybean plants have the ability to develop symbiotic associations with *Bradyrhizobium japonicum* and induce the organogenesis of the highly specialized organs, nodules, where atmospheric dinitrogen is fixed into ammonia which is taken up by the plants as a source of nitrogen. However, low soil temperatures significantly inhibit legume nodulation and nitrogen fixation (Zhang and Smith, 1994), the initial stages being the most sensitive. This reduction in nodulation and nitrogen fixation substantially reduces plant growth and yield. This is particularly important to Canadian conditions where low spring soil temperatures (in the field) prevail during the early stages of legume plant growth and development, the most sensitive stage in the nodulation process. However, rhizobia induced with isoflavonoid (genistein) signals can partially overcome this low temperature induced inhibitory effect on nodulation (Zhang and Smith, 1995), leading to increased legume crop yields under Canadian field conditions (Zhang and Smith, 1996b; 1997). This technology is based on isoflavonoid signals; these compounds are both exceedingly expensive (even when purchased in industrial quantities they are on the order of \$100 g^{-1}) and harmful to the rhizobia being cultured for inclusion in legume inoculants, posing problems for product formulation.

We started this research project with the objective of searching for other compounds that could induce nodulation genes of *Bradyrhizobium japonicum* and, if possible, developing a signal-based-technology for soybean that could replace the already existing flavonoid-based-technology.

Preface to Section 3

Section 3 is comprised of a manuscript by myself, Alfred Souleimanov and Donald L. Smith and has been submitted to the journal *Canadian Journal of Microbiology* for publication. The format has been changed, as much as possible, to be consistent with the rest of the thesis. All literature cited in this section is listed in the references section at the end of the thesis. Tables and figures are presented at the end of this section.

The role of flavonoid inducer molecules in the rhizobia-legume symbiosis is well known. However, recent research findings have demonstrated that some non-flavonoid molecules can also act as *nod* gene inducers in rhizobia. Among the non-flavonoid inducer molecules are the jasmonates. Their potential role in this capacity was only recently reported; Rosas *et al.* (1998) found that this class of signaling molecules can induce nodulation genes of fast growing *Rhizobium meliloti.* Since *B. japonicum* is a slow growing species, physiologically quite different from fast growing rhizobia, I conducted research studies to explore the possibility that jasmonates might induce Nod factor production, the end product of the *nod* genes, by *B. japonicum.* This is the first report demonstrating that exposure to jasmonates causes LCO production by any of the rhizobia.

Section 3

Jasmonates induce Nod factor production by *Bradyrhizobium japonicum*

3.1 ABSTRACT

Jasmonates are signaling molecules involved in induced systemic resistance, wounding and stress responses of plants. They are found in the rhizosphere and can also act as signaling molecules in rhizobia-legume symbioses by inducing nodulation (*nod, nol, noe*) genes. In order to test whether jasmonates can effectively induce the production and secretion of Nod factor (lipo-chitooligosaccharides – LCOs) from rhizobia, we used two *B. japonicum* strains, 532C and USDA3, induced with jasmonate (JA), methyl jasmonate (MeJA) and genistein (Ge). As genistein is well characterized as an inducer of *nod* genes, it was used as a positive control. Our HPLC profile of LCOs isolated following treatment with jasmonates or genistein showed that both JA and MeJA effectively induced *nod* genes and caused production of LCOs from bacterial cultures. JA and MeJA are more efficacious inducers of LCO production than genistein. When added together, genistein and JA or MeJA resulted in greater LCO production than either alone. This enhanced LCO production demonstrates the complexity of inducer molecule perception and transcriptional regulatory mechanisms of *nod* genes in *B. japonicum*. This is the first report that jasmonates induce Nod factor production by *B. japonicum*. This report establishes the role of jasmonates as a new class of signaling molecules in *Bradyrhizobium*-soybean symbiosis.

Keywords: Nod factor, lipo-chitooligosaccharides, jasmonic acid, methyl jasmonate, genistein, *nod* gene induction, *Bradyrhizobium japonicum*

Abbreviations: JA, jasmonic acid; MeJA, methyl jasmonate; HPLC, high-performance liquid chromatography; LCO, lipo-chitooligosaccharide.

3.2 INTRODUCTION

The early events that lead to successful formation of nitrogen fixing nodules are multi-step interactions between rhizobia and their host plants. During the initial stages of this complex inter-organismal interaction, legume plants roots synthesize and exude a diverse array of compounds into the rhizosphere, including the well characterized flavonoid signals (Phillips, 1992). These compounds serve as chemoattractants for rhizobia, influence bacterial growth and selectively induce the expression of nodulation (*nod* – including *nol and noe*) genes of symbiotic rhizobia (Schlaman *et al.*, 1992; Pueppke, 1996). Soybean roots exude isoflavonoids, primarily genistein and diadzein, which are effective inducers of *nod* genes in *B. japonicum* (Kosslak *et al.*, 1987).

The transcriptional regulation of *nod* genes is very complex and varies from species to species and even from strain to strain. However, this process requires the regulatory protein nodD which, after interaction with appropriate flavonoid signal(s) from plants, binds with the *nod* box, a conserved, *cis*-acting promotor element that induces the expression of the *nod* genes. The DNA helix in the vicinity of the *nod* box is deformed so that the adjacent *nod* genes can be transcribed (Fisher and Long, 1993). The product of the induced *nod* genes is the synthesis of lipo-chitooligosaccharides (LCOs) –Nod factors (Carlson *et al.*, 1993). These Nod factors are exported from rhizobia and act as return signals to the plants; they stimulate nodule morphogenesis in the host plants. The bacteria reside inside these nodules as bacteroids and fix atmospheric dinitrogen, which is supplied to the plants in exchange of organic acids (Stougaard, 2000).

Nod factors are composed of three to five 1-4 β-linked N-acetyl glucosamine units with the N-acetyl group of the terminal non-reducing sugar replaced by an acyl chain with 16 or 18 carbons and with varying degrees of saturation. Various "substitutions" at both reducing and non-reducing termini of the chitin backbone are possible, such as methylation, acetylation, carbamoilation or sulfation. Rhizobia produce a range of Nod factors and this diversity is even found in Nod factors of the same rhizobial strain (Stacey *et al.*, 1995). Research efforts during the last decade have made possible the structural identification of many Nod factors from a wide range of rhizobia. These LCOs show

specificity in their signal activity and contribute to host specificity in the rhizobia-legume nodulation process (Spaink *et al.,* 1991, Fisher and Long, 1992; Stougaard, 2000).

Jasmonic acid is a derivative of linolenic acid, and consists of a cyclopentanoic ring where a pentenyl side chain and an acetic acid are attached. It is biosynthesized in plants via the octadecanoid pathway (Vick and Zimmerman, 1984). Jasmonic acid occurs ubiquitously in plants and plays a central role in internal signaling pathways leading to the activation of defense responses of plants in response to insect feeding, pathogen infection and plant responses to environmental stresses (Creelman and Mullet, 1997; Wasternack and Parthier, 1997). The role of JA in multiple aspects of plant growth and physiology has been an attractive area of research. Jasmonic acid also plays key roles in plant development (as reviewed by Creelman and Mullet, 1997), and touch perception of plants (mechanoperception) (Weiler, 1993). It is recognized as a major intermediate in the wound signal transduction cascade (Farmer and Ryan, 1990). Jasmonic acid induces the production of phytoalexins (Blechert *et al.,* 1995) in plants and it has been suggested that JA works as a signal in elicitor-induced biosynthesis of phytoalexins by inducing transcription of the genes coding for phenylalanine ammonia lyase (PAL) (Gundlach *et al.,* 1992) and chalcone synthase (CHS) (Richard *et al.*, 2000).

Besides their well established role in various plant physiological responses, jasmonates have been found in the rhizosphere (Pareek and Guar, 1973). This has been attributed to their rhizosecretion from roots (Dathe *et al.*, 1994). Rosas *et al.,* (1998) reported that these molecules can induce the transcription of *nod* genes in *Rhizobium meliloti* strains. However, the role of jasmonates on the induction of *nod* genes in *Bradyrhizobium japonicum* has not been studied. Since *nod* genes are responsible for the biosynthesis of Nod factors, we conducted this study to test the hypothesis that induction of *B. japonicum* with jasmonates causes the production and excretion of lipo-chitooligosaccharide signal molecules.

3.3 MATERIALS AND METHODS

3.3.1. Chemicals

The inducer compounds tested for the induction of wild type strains of *Bradyrhizobium japonicum* are listed in table 3.1. Genistein (4', 5, 7, trihydroxyisoflavone, purity 98%,), jasmonic acid [(±)-1α-2β-3-oxo-2-[cis-2-pentenyl] cyclo-pentaneacetic acid, $C_{12}H_{18}O_3$], and methyl jasmonate (methyl 3-oxo-2-[2-pentenyl] cyclo-pentaneacetic acid, 95% purity) were obtained from Sigma-Aldrich Chemical Company Inc (Mississauga, ON, Canada).

3.3.2. Bacterial culture and incubation

The two *B. japonicum* strains used in this study were 532C, and USDA3. The bacterial cultures were grown at 28 ± 1 °C in 250 mL flasks containing 100 to 150 mL of yeast extract mannitol (YEM) medium (mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, yeast extract 0.4 g, and distilled water 1000 mL, pH 6.8) (Vincent, 1970), shaken at 150 rpm in the dark. At the mid-log phase, subcultures were started by inoculating 5 mL of the first culture per 250 mL of YEM medium in 4 L flasks containing 2 L medium, or 500 mL flasks containing 250 mL of medium, under conditions described above. When the culture OD_{620} reached 0.2 to 0.4, inducer compounds were added to the culture. Genistein, a well characterized inducer of *B. japonicum* (Kosslak *et al.*, 1987), was used as a positive control. Jasmonic acid and MeJA were added into the individual flasks so that the final concentration for each was 50 μM. Each treatment was replicated four times. The final genistein concentration was 5 μM. All the stock solutions of inducer molecules were made in methanol. After induction, the cultures were incubated for an additional 2 to 3 days and the LCO extracted according to the procedure described below.

3.3.3. Extraction and purification of LCOs

The *B. japonicum* cultures were extracted with 40% HPLC-grade 1-butanol. The cultures were shaken vigorously after addition of 1-butanol, and were then allowed to stand overnight. LCO dissolves into the butanol, which separates as the top phase of the solution; this was carefully removed and condensed in a low-pressure rotary evaporator system (Yamato RE500, Yamato, USA) at 50 °C and a speed of 125 rpm. Evaporation was continued until dryness and the dried extract was resuspended in 4 mL of 18 % acetonitrile.

The extract was loaded on a C-18 column (PRESEP™ Fisher Scientific, Montreal, Canada) and was eluted three times using 10 mL of 30% acetonitrile. We used 60% acetonitrile for a second elution and this eluent contained the Nod factors. Nod factors were further isolated and purified isolated by HPLC. The HPLC was fitted with Waters 501 pumps, a Waters 401 detector set at 214 nm and a WISP712 autosampler using a C18 reverse phase column (0.46 X 25 cm, 5 mM) (Vydac, CA, USA). The chromatography was conducted for 45 min using a linear gradient of acetonitrile from 18 to 60% as described by Souleimanov *et al.* (2002). Identification of various Nod factors (Figure 3.1) was conducted by comparing the retention time of standard (identified by mass spectrometry) Nod factors from strain 532C. In order to quantify individual Nod factors, the concentration of the collected Nod factor(s) was calculated by comparing area under the peak of the standard corresponding LCO, at a known concentration. The individual LCO peaks were collected, rechromatographed and collected again. A sample of this pure material was sent for mass spectrometry analysis (Mass Spectrometry Unit, Montreal Proteomics Network) for chemical structure identification and confirmation. The LCOs collected following each inducer treatment were also tested for their biological activity using root hair deformation as a bioassay, as described in section 3.3.4.

3.3.4. Statistical analysis

Statistical analysis of the data was carried out with analysis of variance (ANOVA) using the Statistical Analysis System computer package (SAS Institute, 1988). Comparisons

among treatment means were made with an ANOVA protected LSD test at the 0.05 level of statistical significance.

3.4 RESULTS

3.4.1 Jasmonates induce LCOs production by *B. japonicum*

The HPLC profile of LCO extract from induced bacterial cultures of both strains showed that jasmonates and genistein induced LCO production from *B. japonicum* cells. *B. japonicum* produces a number of structurally different LCOs (Stacey *et al.*, 1995), however, in this study, we quantified the four LCOs from the induced cultures (Figure 3.1.) that *B. japonicum* cells produce in the greatest abundance. We found that both jasmonates (JA and MeJA) and genistein induced all the four LCOs from induced bacterial cultures (Figure 3.2). However, there was variation among the yields of individual LCOs following induction with JA, MeJA or genistein. Jasmonates preferentially induced LCO (RT 32.24 min, Nod BjV (Ac, $C_{16:0}$, MeFuc) as compared to genistein induced cultures. Genistein induced cultures showed low levels of this LCO (Figure 3.2). The two strains produced different amounts of LCO. Strain USDA3 was more sensitive to inducer molecules, as indicated by greater LCO production, than 532C (Figure 3.3).

3.4.2 Synergism between genistein and jasmonates

Since Nod factor production profile of jasmonate induced cultures was different from that following genistein induction, and given that jasmonates and flavonoids are very different chemically (flavonoids and lipids, respectively), we studied the Nod factor production pattern of the tested strains following exposure to genistein and jasmonates in combination. When the inducer molecules were added to bacterial cultures together, at optimum concentrations for each (5 μM for genistein and 50 μM for JA and MeJA), Nod factor production was greater than when either was used alone. Again strain USDA3 yielded more LCOs than strain 532C (Figure 3.2).

3.5 DISCUSSION

Our knowledge of physiological and molecular aspects of the signaling between rhizobia and legumes has expanded rapidly. Research during the last decade has unraveled this inter-organismal interaction at the molecular and biochemical levels. As we attempt to better understand how this interaction works, new research areas emerge for further exploration. Great advances have been reported regarding the characterization of *nod* gene inducers secreted from legume roots, the regulation of rhizobial *nod* genes and the biosynthesis and secretion of structurally diverse Nod factors from rhizobia. Yet the complete picture of this sophisticated and subtle signaling is still not fully clear. In particular, we remain ignorant of the many aspects of *nod* gene inducer perception, how legume plants might benefit from more than one inducer molecule, Nod factor perception and subsequent signal transduction during the initial stages of nodulation.

A number of *nod* gene inducer compounds secreted from legume roots have been characterized (Stacey *et al.*, 1995). Most of these compounds belong to the flavonoid group, and share a common phenylproponoid biosynthetic pathway. The specificity of these *nod* gene inducers is tightly regulated and only compatible rhizobia respond to specific flavonoid molecules. It was thought that flavonoids were the only plant-to-bacteria signal molecules that induce *nod* genes, however three non-flavonoid classes of *nod* gene inducers have also been identified. These are betaines (stachydrine and trigonelline), secreted from germinating alfalfa seeds (Phillips *et al.*, 1992), aldonic acids (erythronic acid and tetronic acid) rhizosecreted from lupin roots (Gagnon and Ibrahim, 1998) and jasmonates, initially described by Rosas *et al.*, (1998) for *Rhizobium meliloti.*

Since the end product of the *nod* gene induction is LCOs, we induced bacterial cultures with jasmonates and genistein (a positive control). The HPLC profile of the LCOs produced by the two tested strains showed that LCO yield due to jasmonate induction was greater than that produced following induction with genistein. One interesting observation was the greater production of LCOs following treatment of *B. japonicum* cells with genistein and jasmonates, than with either alone (Figures 3.2 & 3.3). The greater effect of genistein and jasmonates together suggests that these two *nod* gene inducers utilize at least different receptors and possibly other down stream elements

involved in signal transduction. It seems likely that the concomitant activation of different regulatory mechanisms enhances transcription of *nod* genes leading to production of greater amounts of LCOs. Bacterial employment of different pathways for *nod* gene induction is well documented in rhizobia. In *R. meliloti*, the non-flavonoid *nod* gene inducing betaines (trigonelline and stachydrine) induce the transcription of *nod* genes via NodD2 but not NodD1 protein. While, 4,4'-dihydroxy-2'-methoxychalcone (MCh), activates *nod* genes by activating both NodD1 and NodD2 in the same bacteria (Phillips *et al.,* 1992). Luteolin, a flavonoid inducer, activates *nodD1* and *nodD3* (Mulligan and Long, 1989) but does not activate *NodD2* (Honma *et al.,* 1990).

The differential induction of *nod* genes through different regulatory pathways and the benefit this might provide to the rhizobial cells is not clearly understood. However, it seems that by evolving different regulatory pathways, rhizobia might benefit maximally at the *nod* gene induction level. One simple explanation of this might be that proteins involved in different regulatory pathways bind to different sets of bacteria-to-plant signaling molecules resulting in maximum induction of *nod* genes. This phenomenon seems to be the case when we induce bacterial cells with jasmonates and genistein together; they result in production of more LCOs than when either is used alone. Synergy among *nod* gene activators has also been described by Begum *et al.* (2001b) who reported that induction by two *nod* gene inducing compounds, hesperatin and naringenin, was more effective than either one alone, although in this case both inducing compounds were flavonoids. A greater effect of the two inducers together was also shown by Gagnon and Ibrahim (1998). They reported that *nod* gene induction activity was higher when a combination of tetronic acid (a non-flavonoid) and luteolin (a flavonoid) was used to induce cultures of *S. meliloti.*

At this time we do not know how jasmonates induce *nod* genes in *B. japonicum*, leading to the production of LCOs, however, the finding that these molecules induce LCOs and that, when added with genistein, they show a greater effect than when added alone, suggests that these two sets of inducer molecules may induce *nod* genes using 1) different receptor proteins, or 2) binding to one receptor protein with different binding sites where different inducer compounds bind to different sites on the receptor, or, 3) possibly there is a single binding site on the regulatory protein, which does not show

specificity and can bind to many *nod* gene inducer molecules, leading to *nod* gene induction and biosynthesis of LCOs. This latter class of receptor would seem somewhat surprising, given the high degree of specificity found in flavonoid mediated *nod* gene induction in rhizobia. The regulation of *nod* gene induction in *B. japonicum* is complex as it involves *nodV*, *nodW* and *nolA*, in addition to the *nodD* gene (Loh *et al.,* 1997; 1999). It will be interesting to explore the interaction of jasmonates and *B. japonicum* using strains with deleted *nodD, nodV, nodW, nolA* and/or all of these regulatory genes, to further dissect the jasmonates mediated complex regulatory pathways of *nod* gene induction in *B. japonicum.*

The initial stages of the N_2 fixing symbiosis are an infection, and so are similar to pathogen attack. Since jasmonates are the major signals involved in wounding responses of plants (Creelman and Mullet, 1997), it will be interesting to investigate the possibility that rhizobia, at the point of entry into plant roots, produce fatty acid precursors of the octadecanoid pathway via membrane lipid breakdown, during the process of wounding, thus leading to the biosynthesis of jasmonates. These jasmonates might then induce rhizobial *nod* genes, thus further enhancing establishment of the symbiosis process. Also, induction of the octadecanoid pathway during the initial stages of nodulation may induce JA mediated resistance in plants to phytopathogens in the absence of salicylic acid (SA). SA biosynthesis is inhibited during the initial stages of symbiosis (Blilou *et al.,* 1999; Martinez-Abarca *et al.,* 1998).

In our study, it was interesting to note that jasmonates preferentially induced some LCOs at much higher levels than genistein (Figures 3.2, 3.3). This suggests that specific rhizobial *nod* genes are differentially activated by jasmonates and flavonoids, supporting the possibility that differences in the perception and response systems for jasmonates and genistein go beyond just the receptor.

Taken together, all these data show that *nod* gene inducer perception, signal transduction, and biosynthesis of LCOs is extremely complex. Even after induction, specific inducer molecules may preferentially produce specific LCOs that are produced in smaller amounts following *nod* gene induction by other inducer molecules. Thus it seems that perception of multiple inducer molecules by rhizobia might have some role in LCO production and thus coordinated application of different inducer molecules may cause

overall maximum production of specific LCOs produced by one strain. The combination of jasmonates and flavonoids could provoke production of a set of LCOs more appropriate to nodulation by a specific legume than induction by flavonoids alone, and could therefore contribute to host-symbiont specificity. It is of ecological importance that plants have already been reported to excrete jasmonates into the rhizosphere (Dathe *et al.*, 1994). It will now be key to determine if the levels produced by soybean seed effusates and root exudates are sufficient for *nod* gene induction.

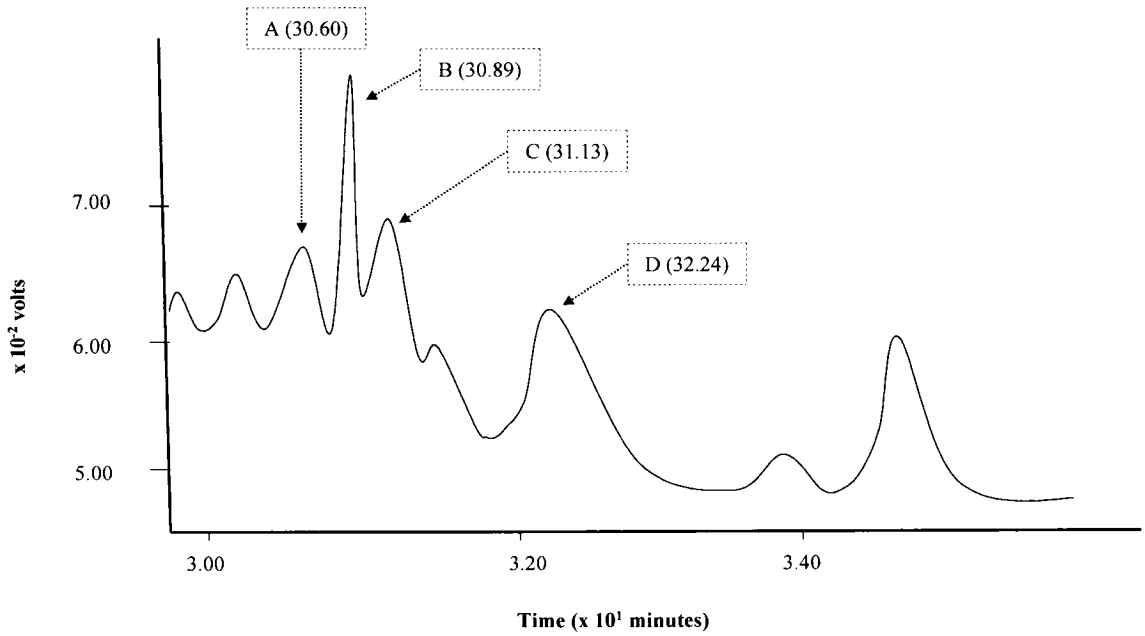
In summary, this is the first report, to the best of our knowledge, that bacterial cultures induced with jasmonates produce LCOs. When measured with HPLC, we found that jasmonates are more effective than genistein at inducing the four LCOs produced in greatest abundance by *B. japonicum*. However, the ratios for the four most commonly produced LCOs were different when induced by jasmonates than when induced by genistein. The combination of genistein and jasmonates at optimum concentrations resulted in the production of greater amounts of LCOs than when either was applied alone. Being effective inducers of LCOs, jasmonates alone or in combination with genistein can be used for increased LCO production from *B. japonicum*. Thus, this report documents the role of jasmonates as a class of signaling molecules in the rhizobia-legume symbiosis.

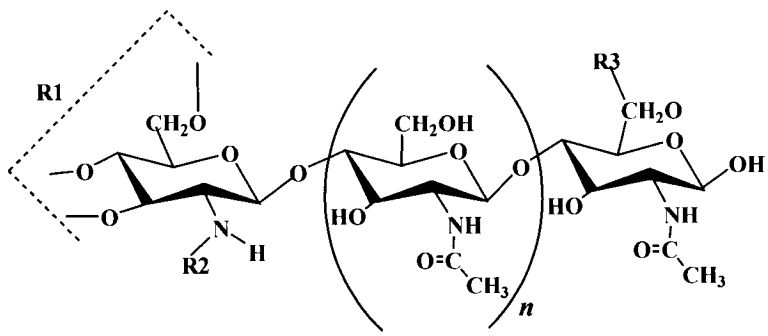
Table 3.1. Inducer compounds tested for induction of wild type strains of *Bradyrhizobium japonicum*, their formulae and trivial and generic names

Compound Name	Formula	F. Weight	Generic Name
Jasmonic acid	$C_{12}H_{18}O_3$	210.3	(±)- 1α, 2β-3-Oxo-2-(cis-2-pentenyl) cyclopentaneacetic acid
Methyl jasmonate	$C_{13}H_{20}O_3$	224.3	Methyl 3-Oxo-2-(2-pentenyl) cyclopentaneacetic acid
Genistein	$C_{15}H_{10}O_5$	270.2	4`, 5,7-Trihydroxyisoflavone

F. Weight: Formula weight

Figure 3.1. Nod metabolites produced by *Bradyrhizobium japonicum*. The basic structure of LCOs (lipo-chitooligosaccharides) has 4-5 *N*-acetylglucosamine units represented by "n". This basic structure can have different substitutions denoted at R1, R2, R3, R4 and R5 (Stacey *et al.,* 1995). The HPLC chromatogram shows different retention time (RT – min) for different LCOs.





Peak	Nod metabolite	RT	n	R1	R2	R3	$(M+H)^+$
A	Nod Bj-V($C_{16:0}$, MeFuc)	30.60	3	H	$C_{16:0}$	2-O-MeFuc	1390
B	Nod Bj-V($C_{18:1}$, MeFuc)	30.89	3	H	$C_{18:1}$	2-O-MeFuc	1416
C	Nod Bj-IV(Cb, $C_{18:1}$, MeFuc)	31.13	2	Cb	$C_{18:1}$	2-O-MeFuc	1432
D	Nod Bj-V(Ac, $C_{16:0}$, MeFuc)	32.24	3	Ac	$C_{16:0}$	2-O-MeFuc	1256

RT = retention time (minutes)

Figure 3.2. LCO production by *B. japonicum* strain 532C and USDA3 cultures induced with genistein (Ge), jasmonic acid (JA) and methyl jasmonate (MeJA). Genistein was used at 5μM concentration while jasmonic acid and methyl jasmonate were used at 50μM concentration. Each value is plotted as the mean ±SE (n=4)

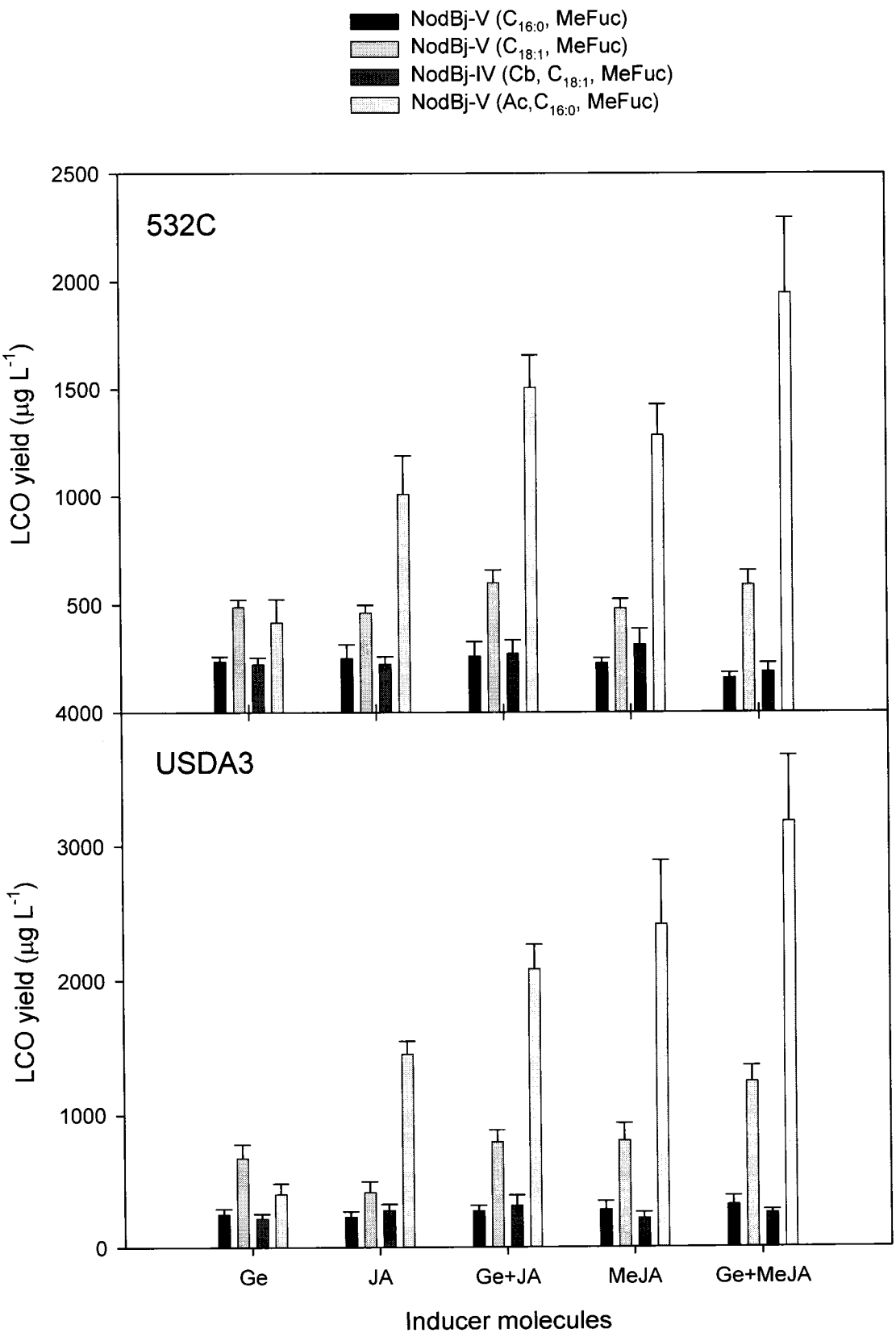
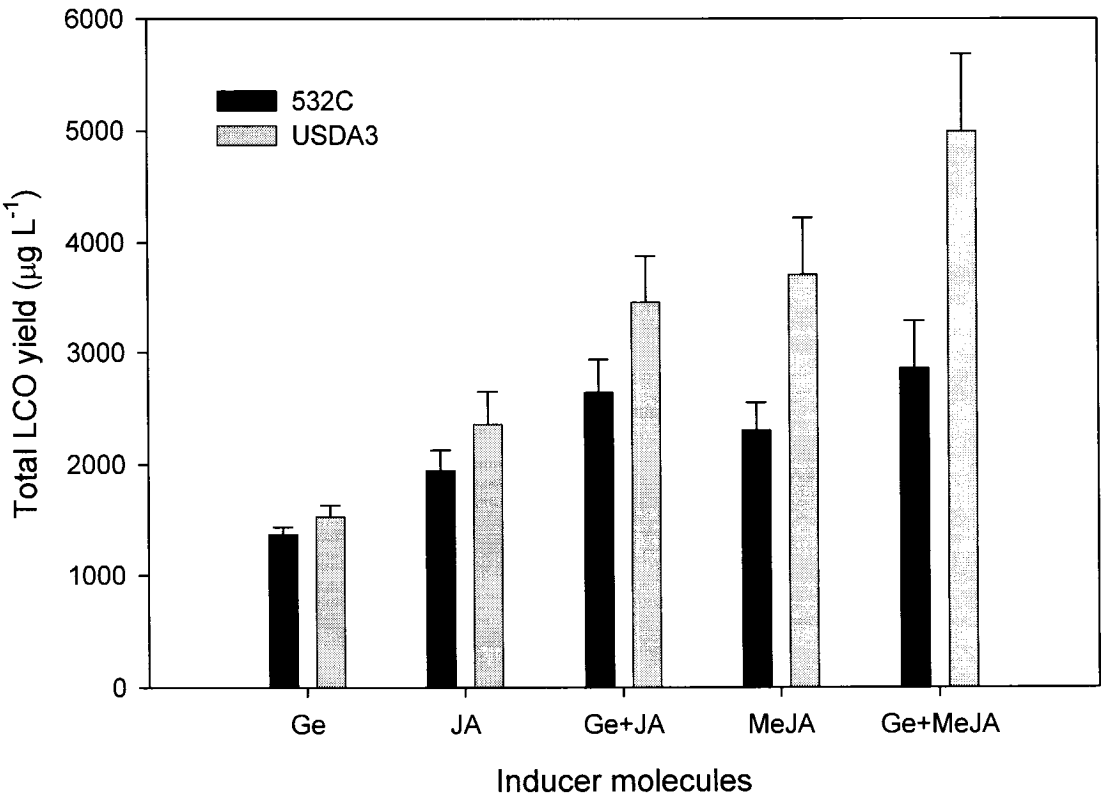

NodBj-V (C16:0, MeFuc)
NodBj-V (C18:1, MeFuc)
NodBj-IV (Cb, C18:1, MeFuc)
NodBj-V (Ac,C16:0, MeFuc)
532C
USDA3
LCO yield (µg L-1)
2500
2000
1500
1000
500
4000
3000
2000
1000
0
Ge
JA
Ge+JA
MeJA
Ge+MeJA
Inducer molecules

Figure 3.3. LCO production by *B. japonicum* strain 532C and USDA3 cultures induced with genistein (Ge), jasmonic acid (JA) and methyl jasmonate (MeJA). Genistein was used at 5 µM concentration while jasmonic acid and methyl jasmonate were used at 50 µM concentration. Each value is plotted as the mean ±SE (n=4)


6000
5000
4000
3000
2000
1000
0
532C
USDA3
Total LCO yield (μg L-1)
Ge
JA
Ge+JA
MeJA
Ge+MeJA
Inducer molecules

Preface to Section 4

Section 4 is comprised of a manuscript by myself, Alfred Souleimanov, and Donald L. Smith and is submitted to the journal *Molecular Plant-Microbe Interactions* for publication. The format has been changed, as much as possible, to one consistent with the rest of the thesis. All literature cited in this section is listed in the references section at the end of the thesis. Tables and figures are presented at the end of this section.

In the previous section, I have reported that jasmonates can induce Nod factor production by *B. japonicum*, thus acting as signal molecules in the *Bradyrhizobium*-soybean symbiosis. Jasmonates are ubiquitously found in plants and are biosynthesized from linolenic acid via the octadecanoid pathway. Since jasmonates are fatty acids derived from linolenic acid, I hypothesized that this fatty acid precursor could also induce the expression of *nod* genes and biosynthesis of LCOs. To prove this, I used *nodY::lacZ* fused *B. japonicum*, induced with different concentrations of linolenic and linoleic acids and measured the expression of *nod* genes using the enzyme β-galactosidase. Lipo-chitooligosaccharides (LCOs) were quantified from a wild type strain of *B. japonicum* using HPLC and mass spectrometry.

Section 4

Fatty acids of the octadecanoid pathway induce *nod* genes of *Bradyrhizobium japonicum*

4.1 ABSTRACT

Jasmonates act as signal molecules in plants and their involvement in wounding and stress responses are well established. Besides their role *in planta*, these compounds can also act as plant-to-bacteria signals. We have previously shown that jasmonates (JA and MeJA) can induce Nod factor production from *Bradyrhizobium japonicum*. Since jasmonates are fatty acids and are biosynthesized from ά-linolenic acid via the octadecanoid pathway, we tested the hypothesis that these octadecanoid pathway precursors of jasmonates can induce *nod* genes of *B. japonicum*. We used *B. japonicum* strain USDA3 harboring a plasmid with a translational fusion between *B. japonicum nodY* and *lacZ* from *Escherichia coli*. Gene expression was measured by quantitative determination of the enzyme β-galactosidase. Our data showed that both linolenic and linoleic acids induced the transcription of *B. japonicum nod* genes; the maximum enzyme activity occurred at 50μM. These fatty acids were less effective at *nod* gene induction (β-galactosidase activity) than genistein and jasmonates. Since the end products of *nod* gene activity are lipo-chitooligosaccharides (LCOs), we also measured LCO production from fatty acid induced bacterial cultures. LCOs were measured by HPLC. When the cultures of a wild type strain (USDA3) were induced with an optimum concentration (50μM) of these fatty acids they produced measurable levels of LCO. To the best of our knowledge, this is the first report documenting the potential role of fatty acids (linolenic and linoleic) as a new class of signaling molecules in the *Bradyrhizobium*-soybean symbiosis.

Key words: Linolenic acid, linoleic acid, β-galactosidase assay, *nod* gene induction, jasmonates, lipo-chitooligosaccharides, root hair deformation, octadecanoid pathway

Abbreviations: LnA, Linolenic Acid; LcA, Linoleic Acid; JA, Jasmonic Acid; MeJA, Methyl Jasmonate; LCO, Lipo chitooligosaccharide; HPLC, High Pressure Liquid Chromatography; 13-LOX, 13-lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; 12-OPDA, 12-oxophytodienoic acid; ONPG, *O*-nitrophenyl-β-galactoside.

4.2 INTRODUCTION

Nodule formation is a complex process. It results from the interaction between rhizobia and legumes and requires 'cross-talk' between the bacteria and the host plants. During the initial events of symbiosis, plant-to-bacteria signal molecules, flavonoids, are exuded by the host plant at low concentrations, which induce the expression of *nod* genes in rhizobia (Zaat *et al.*, 1987; Kosslak *et al.*, 1987). The products of *nod* gene expression are lipo-chitooligosaccharides (LCOs) which are substituted, β 1,4 linked tri-, tetra-, and pentamers of *N*-acetylglucosamine (Bec-Ferte *et al.*, 1994; Lopez-Lara *et al.*, 1995). Various substitutions along the chitin backbone contribute to specificity in the signaling process. Examples of these substitutions are methylation, acetylation, carbamoilation or sulfation (Spaink *et al.*, 1991; Fisher and Long, 1992; Stougaard, 2000). Following production and secretion, LCOs act as bacteria-to-plant signals and stimulate the formation of nodules on host plants. The bacteria reside inside these nodules, in the form of bacteroids, and fix atmospheric dinitrogen into ammonia (Denarie *et al.*, 1992).

The bacteria-to-plant signal molecules (LCOs) are host-specific and the successful activation of inducible bacterial operons depends upon the presence of correct inducer molecules, which have been identified as specific flavonoids (Peters *et al*, 1986; Kosslak *et al.*, 1987, Zaat *et al.*, 1987; Hartwig *et al.*, 1990a; Banfalvi *et al.*, 1988). They are synthesized via the phenylproponoid biosynthetic pathway (Hartwig, *et al.*, 1991), are exuded from legume plant roots into the rhizosphere (e.g. *Glycine max* - Kosslak *et al.*, 1987; *Phaseolus vulgaris* - Hungria *et al.*, 1991; *Lupinus albus* - Gagnon *et al.*, 1995) and initiate the first step in legume-rhizobia symbiosis by stimulating expression of *nod* genes in rhizobia (Kosslak *et al.*, 1987; Hungria *et al.*, 1992). Besides transcriptional regulation of rhizobial *nod* genes, they act as chemoattractants for rhizobia and also enhance bacterial growth (Aguilar *et al.*, 1988; Caetano-Anolles *et al.*, 1988; Kape *et al.*, 1991). Isoflavones, primarily genistein and diadzein, are the best inducers of *nod* genes in *B. japonicum* (Smit *et al.*, 1992), however, genistein is a stronger inducer of *nod* genes in *B. japonicum* than diadzein (Kosslak *et al.*, 1987).

Jasmonic acid (JA) is a lipid-derived octadecanoid compound which occurs naturally in plants. The biosynthesis of JA and related compounds was first described by

Vick and Zimmermann (1984). It is synthesized from ά-linolenic acid through the action of 13-lipoxygenase (13-LOX) catalyzing oxygenation of ά-linolenic acid into 13-hydroperoxy linolenic acid, which is then converted into allene oxide, through the action of allene oxide synthase (AOS). Allene oxide cyclase (AOC) catalyzes the production of 12-oxophytodienoic acid (12-OPDA) following the action of 12-oxo-PDA (Phytodienoic Acid) reductase and three β-oxidation steps, to form JA. The role of jasmonates in plant responses to stress and wounding is well established. They are not only involved in plant growth and development (reviewed by Creelman and Mullet, 1997) but play an important role in osmotic stress responses and defense responses against pathogens and in response to wounding (reviewed by Blechert, *et al.*, 1995). Their involvement as a signal molecule in the induction of induced systemic resistance (ISR) is also well documented (for review, see Creelman and Mullet, 1997). Besides their role *in planta*, these molecules are also reported to be exuded into the rhizosphere (Pareek and Guar, 1973; Dathe *et al.*, 1994), and are able to induce nodulation genes leading to the biosynthesis of Nod factors from *B. japonicum* (Section 3 of this thesis). Since jasmonates are produced from linolenic and linoleic acids via the octadecanoid pathway in plants, we tested the hypothesis that these fatty acids could induce *nod* genes and Nod factor production by *B. japonicum*.

4.3 MATERIALS AND METHODS

Experiment No. 1: Fatty acid precursors of the octadecanoid pathway and induction of *nod* genes of *Bradyrhizobium japonicum*

4.3.1.1. Bacterial strain, growth and incubation:

Bradyrhizobium japonicum strain USDA3, harboring plasmid GG4, which has a translational fusion between *B. japonicum nodY* and *lacZ* of *Escherichia coli* with a tetracycline resistant marker gene, was used in this experiment. The product of *LacZ* is an enzyme, β-galactosidase, whose activity can be easily quantified, thus, in this experiment *nod* gene expression activity due to various inducer molecules was indirectly measured by the amount of β-galactosidase activity.

Bacteria were grown in petri plates containing yeast extract mannitol (YEM) media and inoculation from single colonies was made in 250 mL flasks containing 100 mL YEM medium (10.0 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, and 0.4 g yeast extract dissolved in 1000 mL of distilled water). In order to maintain the plasmid in the strain, tetracycline was added to the bacterial cultures at a concentration of 20 mg L^{-1}. The cultures were shaken at 150 rev. $min.^{-1}$ for 3 to 5 days at 28 °C ± 0.5°C and then subcultured in 400 mL of fresh medium in a 1000 mL flask. The culture was incubated until the OD_{620} reached 0.1 to 0.2. The culture was then divided into tubes (sterilized) containing 5 mL culture media. Stock solutions of various inducers: genistein, linolenic, linoleic, jasmonic and methyl jasmonate were made in ethanol and appropriate amount of these inducers was added into the tubes before addition of bacterial culture (5 mL into each tube). In separate tubes bacterial cultures were added with methanol solutions without any inducer molecules and these acted as control treatments. In the tubes the final concentration of all the inducers was maintained as 5, 10, 25, 50 and 100 μM. Tubes were incubated for 18h after induction and stored at –20°C until β-galactosidase activity was measured.

4.3.1.2. Chemicals

The inducer compounds tested for induction of *Bradyrhizobium japonicum* are mentioned in table 4.1. Linolenic acid (9,12,15-octadecatrienoic acid, purity 99%), linoleic acid (cis-9, cis-12-octadecadienoic acid, purity 99%), genistein (4', 5, 7, trihydroxyisoflavone, purity 98%,) jasmonic acid (jasmonic acid, $C_{12}H_{18}O_3$) and methyl jasmonate (95% purity) were obtained from Sigma-Aldrich Chemical Company Inc. (Mississauga, ON, Canada).

4.3.1.3. β-galactosidase activity assay

The enzyme β-galactosidase is a very important marker for the *lacZ* gene, which encodes this enzyme, and is frequently used as a reporter gene in gene expression experiments, due to its high specificity and stability. In this experiment, we used *O*-nitrophenyl-β-galactoside (ONPG) as a substrate for the enzyme β-galactosidase, which is a colorless compound and is converted into galactose and *O*-nitrophenol in the presence of β-galactosidase. The *O*-nitrophenol is yellow and can be measured by its absorption at 420 nm. The enzyme activity was measured as described by Miller (1972) and modified by Stachel *et al.* (1985). Briefly, 0.5 mL culture was mixed with 2x Z buffer ($Na_2HPO_4.7H_2O$, 16.1 g, $NaH_2PO_4.H_2O$, 5.5 g, KCl 0.75 g, $MgSO_4.7H_2O$ 0.246 g, β-mercaptoethanol 2.7 g, pH 7.0 dissolved in 500 mL water) and 40 µL toluene and vortexed for 10 seconds. The culture was then incubated in a water bath at 37 °C for 30 minutes and then 0.2 mL of *O*-nitrophenyl β-D-galactoside (ONPG) was added at a concentration of 4 mg mL^{-1}. The cultures were incubated again in the water bath until the color of the culture changed. The reaction time was recorded and the reaction was stopped with 1 M Na_2CO_3. (This increases pH of the reaction mixture to 11 and at this pH, β-galactosidase activity is not active.) After centrifugation at 10,000 rpm for 4 minutes, spectrophotometric readings at OD_{420} were recorded. In another cuvette 0.5 mL of bacterial culture was diluted in 2x Z buffer to 1 mL and a spectrophotometric reading was taken at OD_{600}. The β-galactosidase activity was measured (in Miller units) as follows:

β-galactosidase activity = (OD_{420} x 1000)/(OD_{600} x *T* x *V*)

Where;

OD_{420} spectrometric reading that the yellow color absorbs

OD_{600} spectrophotometric reading that the bacteria absorbs

T is the reaction time, and

V is the volume of the bacterial culture used for enzyme assay

Experiment No. 2: Effect of fatty acids on the production of Lipo-chitooligosaccharides (LCOs) by *Bradyrhizobium japonicum*

4.3.2.1. Bacterial culture and incubation

Bradyrhizobium japonicum strain USDA3 was grown at 28 ± 1 °C in yeast extract mannitol (YEM) medium (Vincent, 1970) (mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, yeast extract 0.4 g), and distilled water 1000 mL, pH 6.8, shaken at 150 rpm in the dark. At the mid-log phase of bacterial growth, 2.0 L subcultures were started by inoculating with bacteria from the first culture (5 mL of the first culture per 250 mL of YEM media), until the OD_{620} reached 0.2 to 0.4. At this stage, linolenic acid, linoleic acid, methyl jasmonate, jasmonic acid and genistein were added to individual flasks containing bacterial culture so that the final concentration for linolenic acid, linoleic acid, methyl jasmonate and jasmonic acid was 50 μM while it was 5 μM for genistein. The culture was incubated for an additional 48 to 96 h and the LCO extracted according to the procedure described immediately below.

4.3.2.2. Extraction and purification of LCO

The LCO from the induced bacterial culture was isolated using 0.4 vol. of HPLC-grade 1-butanol by shaking vigorously for 10 to 15 minutes and then allowing the mixture (1-butanol and bacterial culture) to stand overnight. The top organic fraction, containing dissolved LCO molecules in butanol solvent, was then separated and transferred to a 250 mL flask and placed on a rotary evaporator (Yamato RE500, Yamato, USA) operated at

50 °C and a speed of 125 rpm. Evaporation was continued until the flask became dry. The dry extract in the flask was then resuspended in 4 mL of 18% acetonitrile and kept in the dark at 4 °C in a sealed glass vial until use. This constituted the LCO extract; this extract was fractionated by HPLC for LCO quantification.

The extract was loaded on a C-18 column (PRESEP™ Fisher Scientific, Montreal, Canada) and was eluted three times using 10 mL of 30% acetonitrile. We used 60% acetonitrile for a second elution and this eluent contained the Nod factors. Nod factors were further isolated and purified by HPLC. HPLC (equipped with Waters Model 510 HPLC pumps, a Waters 401 detector set at 214 nm and a WISP 712 autosampler, Waters, MA, USA) analysis was conducted with a Vydac C18 reversed-phase column (0.46 X 25 cm, 5 mM) (Vydac, CA, USA) with a flow rate of 1.0 mL min^{-1} and a Vydac guard column. The chromatography was conducted for 45 min using a linear gradient of acetonitrile from 18 to 60% as described by Souleimanov *et al.* (2002). Identification of various Nod factors was conducted by comparing the retention time of standard (identified by mass spectrometry) Nod factors from strain 532C. In order to quantify individual Nod factors, the concentration of the collected Nod factor(s) was calculated by comparing area under the peak of the standard corresponding LCO, at a known concentration. The individual LCO peaks were collected, rechromatographed and collected again. A sample of this pure material was sent for mass spectrometry analysis (Mass Spectrometry Unit, Montreal Proteomics Network) for chemical structure identification and confirmation.

4.3.2.3. Statistical analysis

Statistical analysis of the data was carried out with analysis of variance using the Statistical Analysis System computer package (SAS Institute, 1988). Comparisons among treatment means were made with an ANOVA protected LSD at the 0.05 level of statistical significance.

4.4 RESULTS

4.4.1. Octadecanoid pathway precursors and *nod* gene induction

We studied *B. japonicum* strain GG4 carrying a *nodY::lacZ* fusion; *nod* gene induction due to inducer molecules was measured by the amount of β-galactosidase activity. Our β-galactosidase analyses showed that when the bacterial cultures were induced with fatty acids, linolenic and linoleic acids; they exhibited increase in enzyme activity in a dose dependant manner. The enzyme activity increased with fatty acid concentration up to 50 μM, after which enzyme activity declined. At 100 μM concentration the tested fatty acids showed no induction of *nod* gene activity (Figure 4.2). The concentration of these fatty acids causing the maximum level of *nod* gene transcription in rhizobial strains was much higher (50 μM) than the isoflavonoid genistein, which shows maximum *nod* gene induction activity at 2 to 5 μM (Smit *et al.*, 1992).

It was interesting to note that both linolenic and linoleic acids showed similar pattern of *nod* gene induction. There was no significant difference between the induction level (enzyme activity) at the same concentrations of the tested fatty acids (Figure 4.2). Both increased *nod* gene induction activity, reaching a maximum at 50 μM, with further increases in concentration causing less *nod* gene activity.

4.4.2. *nod* gene induction due to fatty acids, genistein and jasmonates

Our results show that both linolenic and linoleic acids induced *nod* genes of *B. japonicum*. However, the *nod* gene induction due to these tested fatty acids was substantially less than genistein JA and MeJA (Figure 4.3). Interestingly, the linolenic and linoleic acid concentrations causing maximum *nod* gene induction (50 μM) was the same as for JA and MeJA. We have previously shown that at 50 μM concentration, JA and MeJA shows maximum *nod* gene induction activity (Mabood and Smith, unpublished data). Thus, linolenic and linoleic acids seem to be working through the same receptor as MeJA, but less effectively.

4.4.3. Octadecanoid pathway precursors and production of Lipo-chitooligosaccharides (LCOs)

The HPLC analysis of LCO molecules (Figure 4.4) of induced bacterial cells confirmed the β-galactosidase analyses and showed that the tested fatty acids caused LCO production from induced bacterial cultures. The four different LCOs (Figure 4.1) analyzed were present in the fatty acid induced bacterial cultures and were absent in the samples from the control flasks. Our results also demonstrate that there were differences in individual LCO yield due to fatty acid induction (Figure 4.4). NodBj-V (Ac, $C_{16:0}$, MeFuc) was the most abundant LCO while NodBj-IV (Cb, $C_{18:1}$, MeFuc) showed lowest yields (Figure 4.4).

4.5. DISCUSSION

Legume plant roots exude a complex array of compounds into the rhizosphere; perhaps the best known of these are the flavonoids, which have the potential to affect relationships between plants and beneficial or deleterious soil-borne organisms. Flavonoids are the plant compounds that function as transcriptional regulators of the *nod* genes in rhizobia, initiating the first step in signal communication between plants and rhizobia. The flavone luteolin was the first such molecule identified; it was isolated from alfalfa roots and was shown to induce the transcription of nodulation (*nod*) genes in *Rhizobium meliloti* (Peters *et al.*, 1986). Subsequently, researchers identified other flavonoid compounds that induce *nod* genes in rhizobia. The fascinating aspect of these flavonoids is that they do not act as universal inducers of *nod* genes and they differ dramatically in their capacity to induce *nod* genes in rhizobia. The isoflavonoids genistein and diadzein, produced by soybean roots, induce the expression of *nod* genes in *B. japonicum,* however, genistein, is a stronger inducer of *nod* genes in *B. japonicum* than diadzein (Kosslak *et al.*, 1987).

Since most of *nod* gene inducers in rhizobia belong to the structurally diverse group of flavonoids, it was generally believed that only flavonoids act as plant-to-bacteria signal molecules. However, several non-flavonoid plant-to-bacteria signal compounds have been identified (Phillips *et al.*, 1992; Gagnon and Ibrahim, 1998). These compounds induce transcription of *nod* genes in rhizobia demonstrating that compounds outside the flavonoid group can also act as *nod* gene inducers. So far, three non-flavonoid classes of *nod* gene inducers have been identified. They are betaines (stachydrine and trigonelline), released from germinating alfalfa seeds (Phillips *et al.*, 1992); aldonic acids (erythronic acid and tetronic acid) secreted from lupin roots into the rhizosphere (Gagnon and Ibrahim, 1998); and jasmonates (jasmonic acid and methyl jasmonate) which has been shown to induce *nod* genes of *Rhizobium meliloti* (Rosas *et al.*, 1998). These three classes of molecules not only differ structurally but also biosynthetically; their biosynthetic pathways are completely different from each other, whereas the various active flavonoid compounds share the common phenylproponoid biosynthetic pathway.

At optimum concentrations *nod* gene induction by linoleic and linolenic acids was found to be weaker than the optimal concentrations of either genistein or methyl

jasmonate, when measured by β-galactosidase activity (Figure 4.2). These results were confirmed at the level of LCO production. Both fatty acids, linolenic and linoleic acids, induced bacterial cultures produced LCOs (Figure 4.4). Fatty acids induced the production of all four studied Nod factors NodBj-V ($C_{16:0}$, MeFuc), NodBj-V ($C_{18:1}$, MeFu), NodBj-IV (Cb, $C_{18:1}$, MeFu), and NodBj-V (Ac, $C_{16:1}$, MeFu). The weaker induction of *nod* genes suggests that these fatty acids interact with a receptor or receptors differently from MeJA and genistein.

We don't know whether these fatty acids induced *nod* genes in a fashion similar to genistein or not, however, it will be interesting to understand the mechanism of *nod* gene induction by these fatty acids. Since *nod* gene induction in *B. japonicum* is fairly complex, as compared to other rhizobia, and involves additional regulatory pathways including *nodV*, *nodW* and *nolA* in addition to *nodD* (Stacey *et al.*,1997; Sanjuan, *et al.*, 1994; Gottfert *et al.*, 1990), further investigation of *nod* gene regulation by these fatty acid molecules will help us understand how these molecules regulate *nod* gene induction in *B. japonicum*.

It has been demonstrated that the isoflavonoids genistein and diadzein induce *nod* genes at very low concentrations (Kosslak *et al.*, 1987). The maximum *nod* gene induction activity for genistein is in the range of 2 to 5 μM (Smit *et al.*, 1992). However, our previous study with jasmonates (Section 3 of this thesis) and the current study with fatty acids show that maximum *nod* gene induction for these compounds (all lipids) was observed when the cultures were induced with 50 μM solutions. Thus, that these fatty acids seem to act similarly to jasmonates in terms of concentration dependent induction of *nod* genes with a maximum at 50 μM. It seems that non-flavonoid molecules are active at much higher concentrations than flavonoid molecules. Similar results have been reported by Gagnon and Ibrahim (1998), who found that non-flavonoid inducers have maximum activity at concentrations much higher than flavonoids. This could mean different receptors or less effective binding at the same receptor.

Linolenic and linoleic acids were poor inducers of *nod* genes, as measured by β-galactosidase activity (Figures 4.3), however that they followed the same pattern of concentration dependent *nod* gene induction as jasmonates strengthens our belief in the finding that these molecules may induce *nod* genes via the same receptor mechanism by

binding to different sites to activate *nod* genes. We don't know why these molecules are relatively ineffective at inducing *nod* genes; however, the fact that these fatty acids can act as signaling molecules and *nod* gene inducers is extremely interesting. This demonstrates that there are a wide range of potential rhizobial *nod* gene inducers, and precursors of the octadecanoid pathway can now be included among these. That such a wide range of compounds can be effective suggests a number of possibilities: 1) multiple receptors, 2) a single receptor with a several binding sites and 3) a single binding site that is not very specific. That latter would seem somewhat surprising, given the high degree of specificity seen in binding of flavonoids.

Since linolenic acid and linoleic acids can be produced via membrane lipid breakdown, during the process of wounding, and that rhizobial infection during the early stages of symbiosis is similar to wounding, it may be that, during the initial processes of nodulation, rhizobial infection triggers plant biosynthesis of these compounds via the octadecanoid pathway, along with the jasmonates, and this may serve to further activate the *nod* genes at the time of first contact between the two potential symbionts.

In summary, to our knowledge, this is the first report demonstrating that precursors of the octadecanoid pathway, linolenic and linoleic acids, can induce rhizobial *nod* genes and cause production of LCOs by *B. japonicum*. Although these fatty acids induced *nod* genes, their induction of *nod* genes was lower than other previously known *nod* gene inducers, genistein and jasmonates; they did induce production of the same suite of LCOs. Thus, this report establishes the role of the octadecanoid pathway precursors as signal molecules that can induce the transcription of *nod* genes and cause Nod factor production in *B. japonicum.*

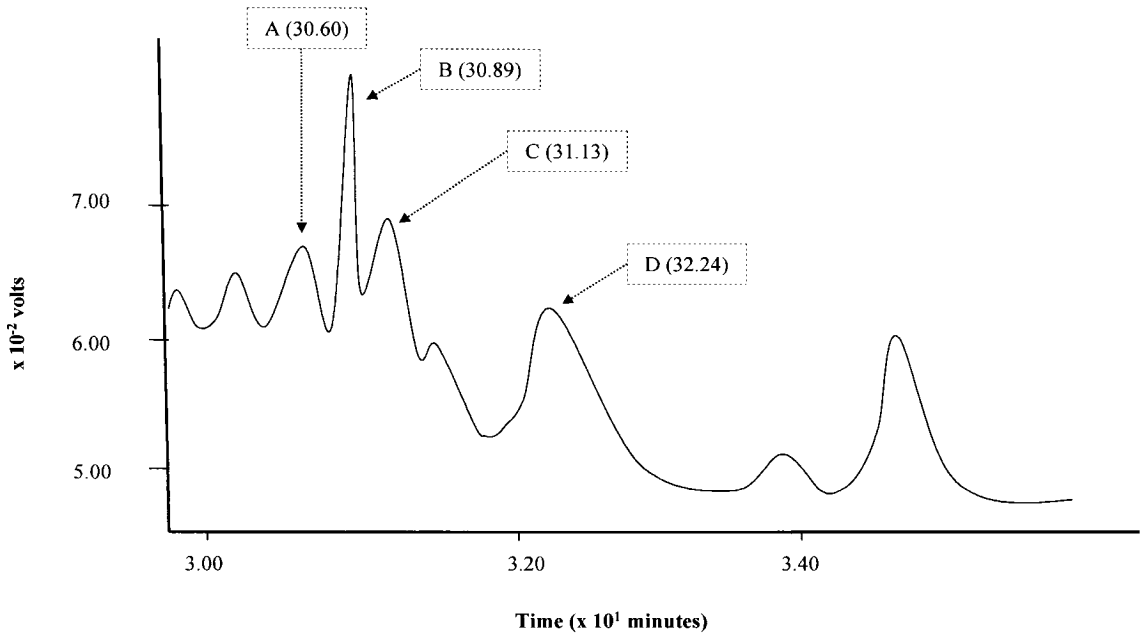
Table 4.1. Inducer compounds tested for *nod* gene induction of *Bradyrhizobium japonicum* strain USDA 3, their formulae, trivial and generic names

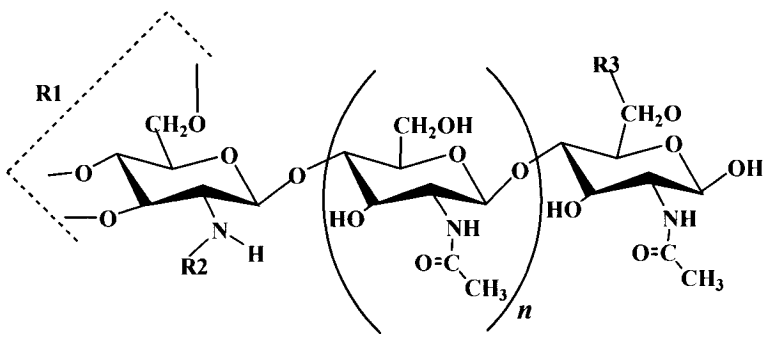
Compound Name	Formula	M. Weight	Generic Name
Linolenic acid	$C_{18}H_{30}O_2$	278.4	cis,cis,cis- 9, 12, 15-Octadecatrienoic acid
Linoleic acid	$C_{18}H_{32}O_2$	280.5	cis,cis- 9, 12-Octadecadienoic acid
Jasmonic acid	$C_{12}H_{18}O_3$	210.3	(±)- 1α, 2β-3-Oxo-2-(cis-2-pentenyl) cyclopentaneacetic acid
Methyl jasmonate	$C_{13}H_{20}O_3$	224.30	Methyl 3-Oxo-2-(2-pentenyl) cyclopentaneacetic acid
Genistein	$C_{15}H_{10}O_5$	270.2	4`, 5,7-Trihydroxyisoflavone

S. # = Serial number

M. Weight = Formula weight

Figure 4.1. Nod metabolites produced by *Bradyrhizobium japonicum*. The basic structure of LCOs (lipo-chitooligosaccharides) has 4-5 *N*-acetylglucosamine units represented by "n". This basic structure can have different substitutions denoted at R1, R2, R3, R4 and R5 (Stacey *et al.,* 1995). The HPLC chromatogram shows different retention time (RT – min) for different LCOs.





Peak	Nod metabolite	RT	n	R1	R2	R3	$(M+H)^+$
A	Nod Bj-V($C_{16:0}$, MeFuc)	30.60	3	H	$C_{16:0}$	2-O-MeFuc	1390
B	Nod Bj-V($C_{18:1}$, MeFuc)	30.89	3	H	$C_{18:1}$	2-O-MeFuc	1416
C	Nod Bj-IV(Cb, $C_{18:1}$, MeFuc)	31.13	2	Cb	$C_{18:1}$	2-O-MeFuc	1432
D	Nod Bj-V(Ac, $C_{16:0}$, MeFuc)	32.24	3	Ac	$C_{16:0}$	2-O-MeFuc	1256

RT = retention time (minutes)

Figure 4.2. Effect of different concentrations of linolenic (LnA) and linoleic (LcA) acids on the induction of *nod* genes of *Bradyrhizobium japonicum* strain USDA 3. Each value is plotted as the mean ±SE (n=3)

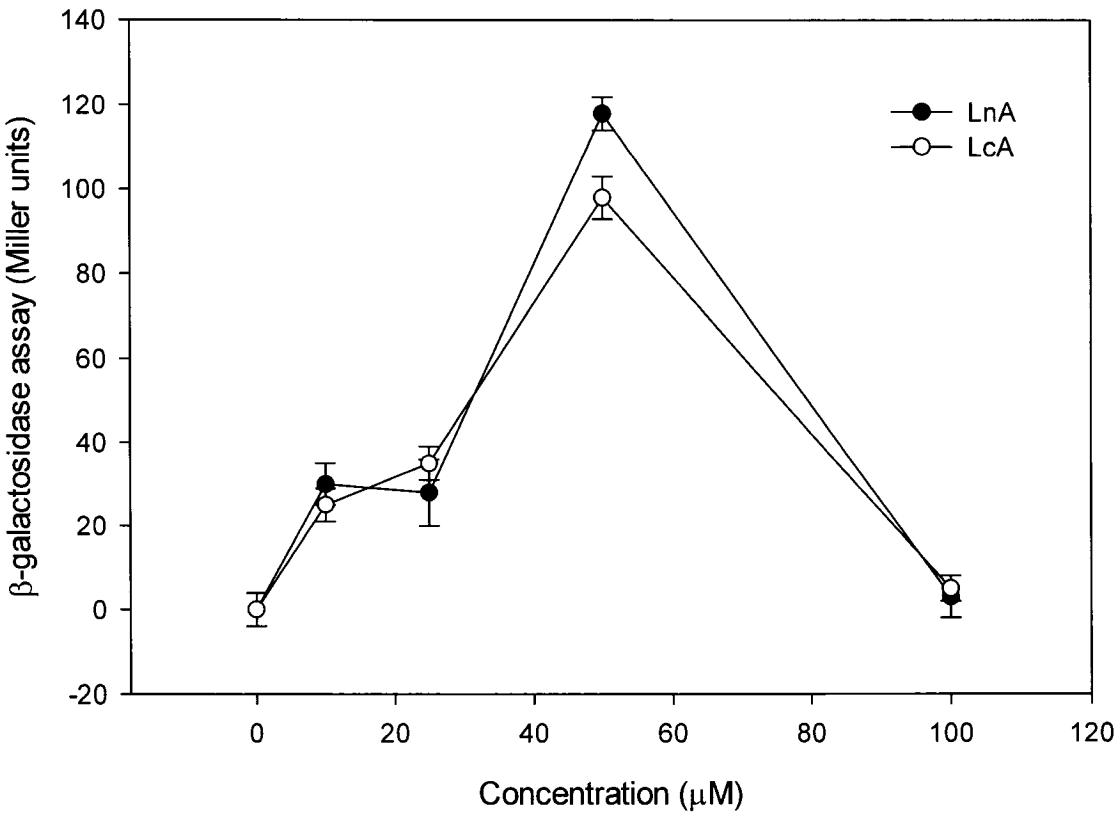

140
120
100
80
60
40
20
0
-20
β-galactosidase assay (Miller units)
0
20
40
60
80
100
120
Concentration (μM)
LnA
LcA

Figure 4.3. *nod* gene induction by linolenic acid (LnA), linoleic acid (LcA), genistein (Ge), jasmonic acid (JA), and methyl jasmonate (MeJA). Genistein was applied at 5 μM while all other inducer molecules were tested at 50 μM concentration. *Bradyrhizobium japonicum* strain GG4 carrying *nodY::lacZ* fusion was used in the study and the gene expression activity was measured by the amount of β-galactosidase activity. Each value is plotted as the mean ±SE (n=3)

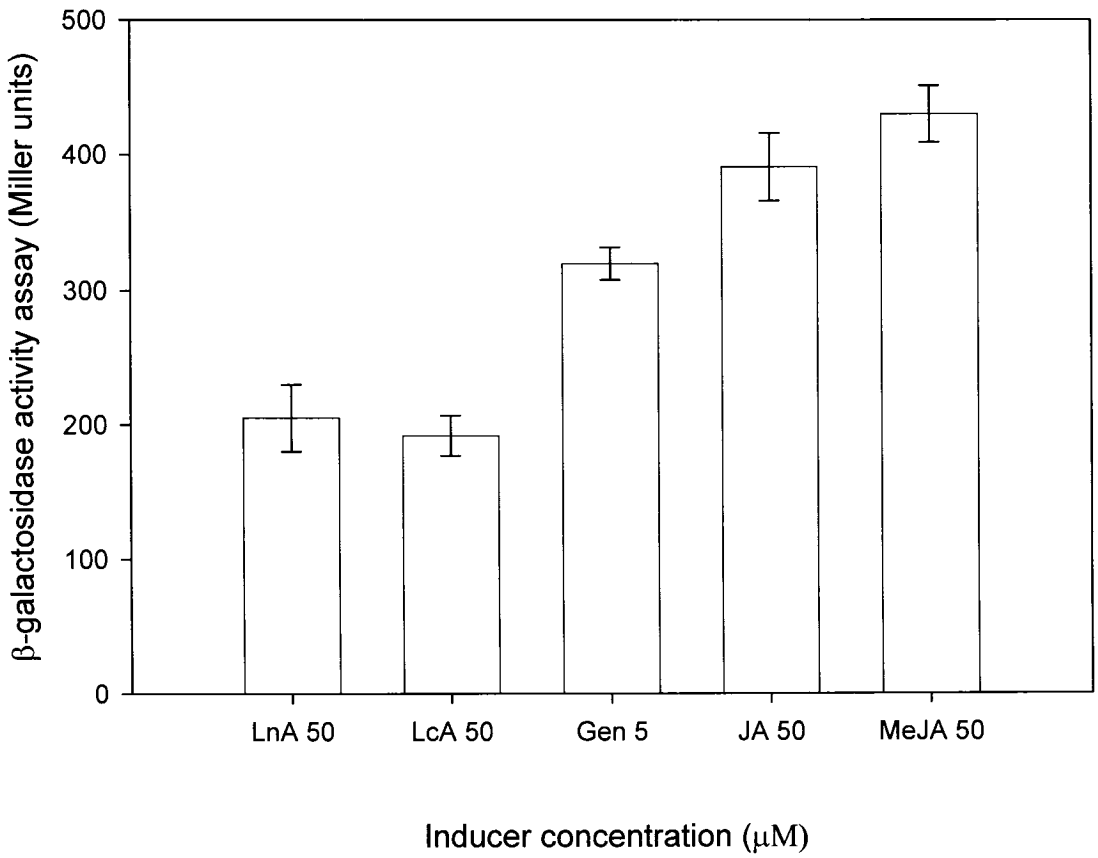
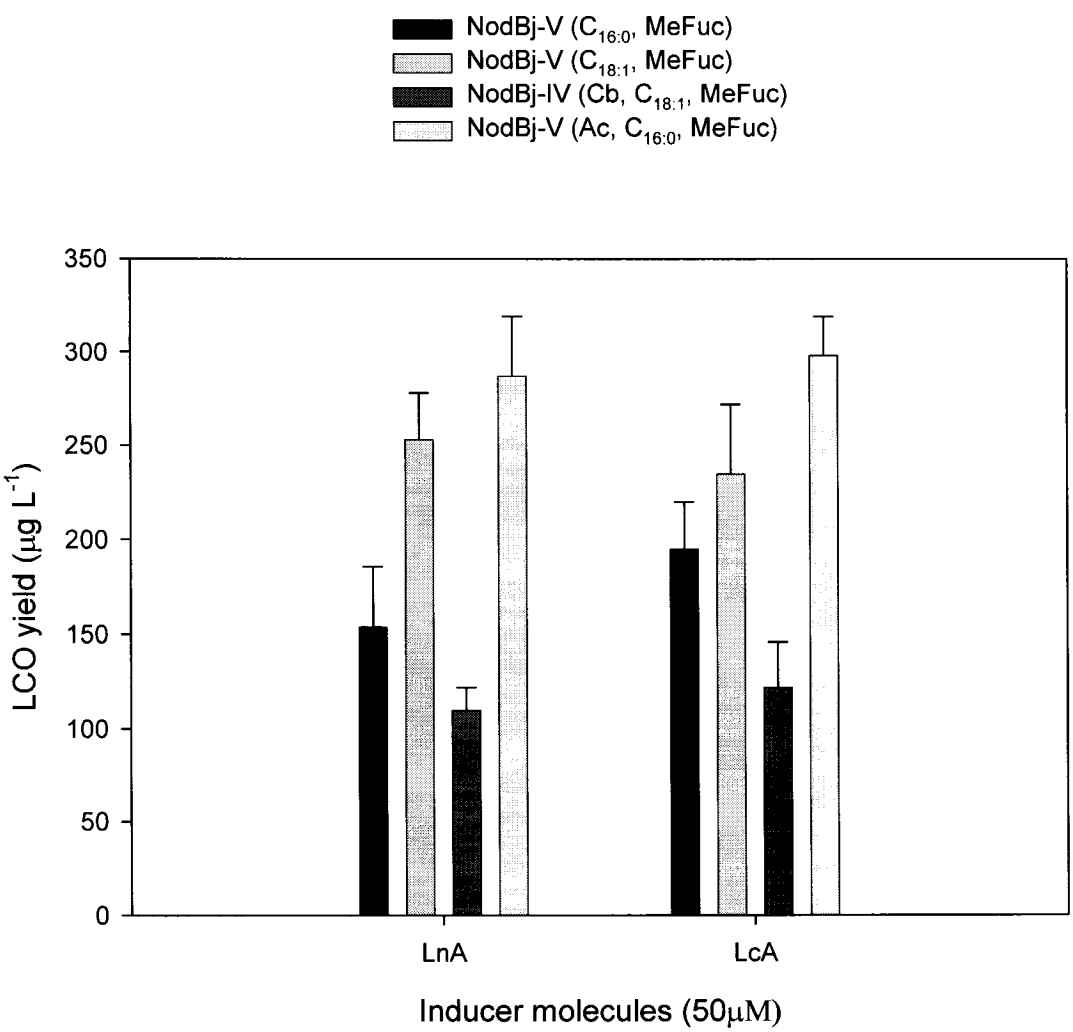

500
400
300
200
100
0
β-galactosidase activity assay (Miller units)
LnA 50
LcA 50
Gen 5
JA 50
MeJA 50
Inducer concentration (μM)

Figure 4.4. Lipo chitooligosaccharide (LCO) production from *B. japonicum* strain USDA 3 cultures induced with linolenic (LnA) and linoleic (LcA) acids. Each value is plotted as the mean ±SE (n=3).


NodBj-V ($C_{16:0}$, MeFuc)
NodBj-V ($C_{18:1}$, MeFuc)
NodBj-IV (Cb, $C_{18:1}$, MeFuc)
NodBj-V (Ac, $C_{16:0}$, MeFuc)
LCO yield (µg L^{-1})
350
300
250
200
150
100
50
0
LnA
LcA
Inducer molecules (50µM)

Preface to Section 5

Section 5 is comprised of a manuscript prepared by myself and Donald Smith and has been accepted for publication in *Physiologia Plantarum*. The format has been changed, as much as possible, to be consistent with the rest of the thesis. All literature cited in this section is listed in the references section at the end of the thesis. Tables and figures are presented at the end of this section.

Low RZT interferes with early inter-organismal signal exchange between the two symbionts, delays nodule organogenesis and the onset of nitrogen fixation in soybean plants. Low RZT inhibits the biosynthesis and rhizosecretion of genistein from soybean roots (Zhang and Smith, 1996a), and the induction of bacterial *nod* genes (Zhang *et al.*, 1996) leading to reduced Nod factor production (McKay and Djordjevic, 1993). Genistein pre-incubated bacterial inoculants applied to seedlings under controlled environment conditions partially alleviated the detrimental effects of low RZT on soybean nodulation and nitrogen fixation (Zhang and Smith, 1995).

In the previous sections, I showed that jasmonates induced Nod factor (LCO) production from *B. japonicum* and when applied simultaneously with genistein showed synergism (Section 3 of this thesis). Since genistein induced *B. japonicum* improves nodulation and nitrogen fixation, in this section, I tested the ability of *B. japonicum* pre-induced with JA, MeJA alone or in combination with genistein to increase nodulation and nitrogen fixation in soybean at optimal and suboptimal RZTs under controlled environment conditions.

Section 5

Pre-incubation of *Bradyrhizobium japonicum* with jasmonates accelerate nodulation and nitrogen fixation in soybean (*Glycine max* (L.) Merr.) at optimal and sub-optimal root zone temperatures

5.1. ABSTRACT

Biological nitrogen fixation by rhizobia-legume symbioses is the most important source of fixed nitrogen in agricultural fields. At the same time, this complex biological system is severely affected by environmental stresses. Low root zone temperature (RZT) negatively affects soybean nodule formation and function. We have previously shown that jasmonates can induce Nod factor production from *B. japonicum* and as such constitute new class of signaling molecules in the *Bradyrhizobium*-soybean symbiosis. We conducted an experiment to study the role of jasmonic acid and methyl jasmonate induced *B. japonicum* on soybean nodulation and nitrogen fixation under optimal (25°C) and suboptimal (17°C) RZT conditions. *Bradyrhizobium japonicum* cells were grown in liquid YEM media and induced with different concentrations of genistein, jasmonic acid, methyl jasmonate or without any inducer added (control). Soybean seedlings were grown at 25°C or 17°C RZT with a constant air temperature (25°C) and inoculated, at the VC (vegetative cotyledonary) stage, with various *B. japonicum* treatments. Our results show that the addition of genistein or jasmonates to *B. japonicum*, prior to inoculation, partially alleviated the low RZT induced inhibitory effects on nodulation and nitrogen fixation. A higher concentration of genistein was inhibitory at 25°C while this same concentration was stimulatory at 17°C. Interestingly, jasmonates pre-induced *B. japonicum* enhanced soybean nodulation and nitrogen fixation at both RZT regimes. The results of this study show that jasmonates constitute a new class of signaling molecules in *B. japonicum*-soybean symbiosis, and that pre-induction of *B. japonicum* with jasmonates can be used to enhance soybean nodulation and nitrogen fixation under both optimal and suboptimal RZT.

Key words: Soybean [*Glycine max*], nodulation, nitrogen fixation, *Bradyrhizobium japonicum*, genistein, jasmonates, low root zone temperature

Abbreviations: RZT – root zone temperature, JA – jasmonic acid, MeJA – methyl jasmonate, cJ - *cis*-jasmone, Ge – genistein, LOX – Lipoxygenase, AOC - allene oxide cyclase, AOS - allene oxide synthase, 12-OPDA - 12-oxo-phytodienoic acid, JMT - jasmonic acid carboxyl methyltransferase, SPAD - Soil Plant Analysis Device

5.2. INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] has the ability to establish a nitrogen fixing symbiosis with *Bradyrhizobium japonicum* and in eastern Canada field grown soybean can fix 100-200 kg N ha^{-1} yr^{-1} (Smith and Hume, 1987). Soybean is a crop of tropical to subtropical origin and as such requires root zone temperatures (RZTs) in the range of 25 to 30°C for optimal growth and production (Jones and Tisdale, 1921). In Eastern Canada, early vegetative growth of soybean occurs at lower RZTs (suboptimal for plant growth), however, the shoot temperature is often elevated. During the first month of the growing season in Canadian soybean production areas, soil temperatures (at 10 cm depth) are often below 15°C and may be below 20°C until July (Lynch and Smith, 1994). This suboptimal RZT seriously inhibits and delays soybean nodulation and nitrogen fixation.

Suboptimal RZT restricts and delays nodule development and function (Lynch and Smith 1994) and this delay is temperature dependent. For instance, the time between inoculation and the onset of nitrogen fixation increases 2-3 days per °C as RZT drops from 25°C to 17°C, while it increases about 7 days per °C when RZT declines below 17°C (Zhang and Smith, 1994; Zhang *et al*, 1995).

Studies with soybean subjected to low RZT have revealed that the inter-organismal signaling between the two symbiotic partners is strongly and negatively affected by low RZTs. It inhibits genistein production and rhizosecretion from soybean roots (Zhang and Smith, 1996). Genistein is an important signal molecule that induces the transcription of nodulation *(nod)* genes in *Bradyrhizobium japonicum*. On the other hand, it also inhibits the transcription of *nod* genes by some rhizobia (Zhang *et al.,* 1996). This disruption in signal exchange between the two partners inhibits the initial events leading to nodule formation, subsequent nitrogen fixation and growth of the plants.

In order to overcome low RZT induced inhibitory effects on soybean nodulation and nitrogen fixation, Zhang and Smith (1995) have shown that nitrogen fixation and nodule development of soybean can be accelerated by inoculation with genistein preinduced *B. japonicum*. Genistein (Ge), an isoflavone, is the major component of soybean seed and root exudates. It induces the *nod* genes of *B. japonicum* (Kosslak *et al.,* 1987). While low RZTs interfere inter-organismal signaling between the two symbiotic

partners, pre-incubation of *B. japonicum* with genistein induced the transcription of *nod* genes partially overcame the detrimental effects of low RZT (Zhang and Smith, 1995).

Jasmonates are a unique class of signal molecules internal to plants. It and related compounds are produced by the octadecanoid pathway. Many of these compounds have diverse biological functions. Jasmonates are important intracellular regulators of diverse developmental processes, such as seed germination, flower and fruit development, leaf abscission, and senescence (Creelman and Mullet, 1995). Jasmonic acid is an essential component required for male fertility in *Arabidopsis* (Feys *et al.,* 1994; McConn and Browse, 1996; Sanders *et al.,* 2000; Stintzi and Browse, 2000). Jasmonic acid and its cyclopentenone precursor OPDA play a central role in plant defense responses against insect and fungal pathogens (Stintzi, *et al.,* 2001). The biosynthesis of JA starts with α-linolenic acid, derived from chloroplast membranes (Ishiguro *et al.,* 2001) or glycerolipids, which might also function as a supply of 12-oxo-phytodienoic acid (12-OPDA) (Stelmach *et al.,* 2001). Cellular organelles such as plastids or peroxisomes are regarded as the primary site of JA biosynthesis. The enzyme 13-lipoxygenase (LOX) converts α-linolenic acid to 13-hydroperoxylinolenic acid, which is further catalyzed by allene oxide synthase (AOS) and allene oxide cyclase (AOC) leading to the biosynthesis of 12-oxo-phytodienoic acid (12-OPDA). 12-OPDA itself acts as an elicitor of defense responses (Stintzi *et al.,* 2001) or can further be reduced by the enzyme 12-OPDA reductase followed by three steps of β-oxidation to produce JA (Schaller 2001). The enzyme jasmonic acid carboxyl methyltransferase (JMT) is responsible for the conversion of JA into its volatile counterpart MeJA (Seo *et al.,* 2001).

One remarkable feature of jasmonates is that some of them can act as signal molecules outside of plants. The volatile member of jasmonate pathway *cis*-jasmone (cJ) or (*Z*)-jasmone is released from plant leaves due to damage such as insect herbivory and may help repel herbivorous species and attract their predators (Birkett *et al.,* 2000). Besides plant-insect communication, methyl jasmonate (MeJA), a volatile derivative of JA, acts as a signal in inter-plant communication among leaves of different plant species, allowing one plant species to activate the expression of defensive genes in a second species (Farmer and Ryan, 1990). Besides their *ex-planta* roles in plant-insect

communication and inter-plant communication, jasmonates can also play a role in rhizobia-legume symbioses.

We have shown that JA and MeJA induce Nod factor, the end product of nodulation genes, production from *B. japonicum* and are even stronger inducers of the *nod* genes than the isoflavone Ge (Section 3 of this thesis). Jasmonates also induced the production of substantially higher amount of LCOs (Section 3 of this thesis) suggesting a potentially important role for these compounds in the *Bradyrhizobium*-soybean symbiosis. In this section, we designed experiments to investigate the effects of *B. japonicum* pre-incubated with JA and MeJA on soybean nodulation and plant growth under optimal and suboptimal RZT conditions.

5.3. MATERIALS AND METHODS

5.3.1. Plant material and controlled temperature conditions

Seeds of soybean [*Glycine max* (L.)] cv. OAC Bayfield were surface sterilized in sodium hypochlorite (2% solution) for 3 min, and then rinsed several times with distilled water (Bhuvaneswari *et al.*, 1980). The cultivar was selected as it was developed for production under short season, cool conditions of eastern Canada and also because it performs well under these environmental conditions. Seeds were sown in trays containing sterilized vermiculite (Applied Industrial Materials Corp., Deerfield, IL). Seven-day-old seedlings at the vegetative cotyledonary stage [VC, unifoliate leaves unrolled sufficiently that the edges were not touching (Fehr *et al.*, 1971)] were transplanted into sterilized pots (1000 mL capacity) containing a mixture of Turface and sand (2:1, v/v)] and were maintained on a growth bench (model GB48, Controlled Environments Ltd., Winnipeg, Manitoba, Canada) at an irradiance of 300 μmol m^{-2} s^{-1} for a 16:8 hours (day:night) photoperiod with a constant air temperature of 25°C.

In order to control the RZTs of the plants growing in pots, the pots were placed in plastic tanks with bottom of the pots sealed to the tank (68 x 42 cm) bottom and circulating cooled water of the desired temperature (17 or 25 ± 1.0 °C) around the pots. There were 10 pots in each tank. In order to facilitate water drainage from the pots, a hole was drilled in the tank bottom below each pot.

After transplanting the plants were acclimatized for 24 h prior to inoculation with symbiotic rhizobial cells. Seedlings were inoculated with induced *B. japonicum* as described below. Modified nitrogen-free Hoagland's solution (Hoagland and Arnon, 1950) was used as a nutrient source for the plants in which the $Ca(NO_3)_2$ and KNO_3 are replaced with 1 mM $CaCl_2$, 1 mM K_2HPO_4, and 1 mM KH_2PO_4 to provide nitrogen free nutrient solution. Before watering the plants with Hoagland's solution, the temperature of the solution was adjusted to the corresponding RZT.

5.3.2. Bacterial culture, induction and inoculation

Bradyrhizobium japonicum strain USDA 3 was cultured in Yeast Extract Mannitol (YEM) media in 500 mL flasks and shaken at 150 rpm at 25°C. After five days, the subculture (approximately with OD_{600} = 0.4) was diluted to an OD_{600} of 0.08 (approximately 10^8 cells mL^{-1}) (Bhuvaneswari *et al.*, 1980) and divided among nine flasks, each flask representing one treatment. The inoculant treatments were: *B.* japonicum without added inducer (control), *B. japonicum* induced with Ge (5, 10 and 20μM), and *B. japonicum* induced with JA or MeJA (20, 30 and 50μM). Inducer molecules were added to the cultures under sterile conditions. The 50 μM concentration for MeJA was used since *B. japonicum* strains, with a reporter gene attached to a *nod* gene promoter, showed maximum *nod* gene induction activity (β-galactosidase activity) (Mabood and Smith, unpublished data) and Nod factor production (Section 3 of this thesis) at this concentration. The cultures were incubated on a shaker, as described above. After 24h of induction and incubation, the bacteria were applied to the soybean seedlings at different temperature regimes. Each seedling received 1 mL of bacterial inoculant which was applied at its base.

5.3.3. Data collection and analysis

Plants were harvested at R4 developmental stage [full pod - pod is ¾ inch long at one of the four uppermost nodes on the main stem with a fully developed leaf (Fehr *et al.*, 1971)] and data were collected on the following variables: nodule number per plant, nodule weight per plant, plant height, number of leaves per plant, leaf area, plant dry weight, and time to leaf re-greening (Zhang *et al.*, 1995). Time to leaf re-greening is a visual indicator of nitrogen fixation in soybean plants. Leaf nitrogen status was indirectly measured using a SPAD (Soil Plant Analysis Device) leaf greenness meter (SPAD-502, Minolta, Japan). The SPAD data were collected starting from inoculation until harvest at different intervals. The plants were allowed to grow for 60 days after transplanting and were then harvested for data collection. At harvest, the plants were also analyzed for nitrogen analysis. The plant nitrogen content was measured using an automated elemental

analyzer (Model NC 2500, CE Instrument Inc., Milan, Italy). The total amount of fixed nitrogen per plant was calculated by the total plant nitrogen content minus the amount of nitrogen in the original seed (11.5 mg $seed^{-1}$; average of 25 seeds). The data obtained were statistically analyzed using CoStat software, and means comparisons were conducted using an ANOVA protected LSD ($p < 0.05$) test.

5.4. RESULTS AND DISCUSSION

5.4.1. Effect of jasmonates on nodulation and nodule mass

Our results show that both JA and MeJA increased both nodule number and weight at both RZTs (Fig. 5.1, 5.2). The effect of JA and MeJA was concentration dependent; both compounds caused maximum increases in nodule number and weight at the highest concentration tested (50 μM). The general pattern of response to concentration was the same at both 25 and 17°C RZTs. Genistein, at 25°C, increased nodule number and weight at 5 and 10 μM, but further increase in concentration decreased nodule number and weight. At 17°C, 20μM genistein caused the largest increase in nodule number and weight (Fig. 5.1, 5.2). Our results with genistein support previous findings of Zhang and Smith (1995) and Pan *et al.,* (1998). Zhang and Smith (1995) reported that pre-incubation of *B. japonicum* cells with genistein increased soybean nodulation. Our finding that the highest tested concentration of genistein (20 μM) was inhibitory at 25°C but increased nodule number at 17°C is also in agreement with Zhang and Smith (1995). Although the mechanism of temperature dependent Ge enhancement of nodulation is not clearly understood there are several reasonable explanations. First, low temperature inhibits the biosynthesis and rhizosecretion of genistein from soybean roots (Zhang and Smith, 1996) and thus addition of Ge might be needed to offset reduced production by roots. Second, at low RZT bacterial cells are less responsive to inducer molecules (Zhang *et al.,* 1996) and therefore higher concentrations of inducer might be needed to maximally induce the *nod* genes of the bacteria.

Genistein acts as a plant-to-bacteria signal molecule and induces the transcription of *nod* genes of *B. japonicum* (Kosslak *et al.,* 1987). The products of the *nod* genes are lipo-chitooligosaccharides (LCOs), which serve as important bacteria-to-plant signal molecules during the early stages of nodulation (Carlson *et al.,* 1993). We have recently discovered that jasmonates are a class of signal molecules that can induce Nod factor from *B. japonicum* (Section 3 of this thesis). This is probably the reason that jasmonate induced bacterial cells enhanced nodulation and nodule dry weight of soybean plants, similar to Ge. However, the difference in responses to concentration ranges of the three

inducers at optimal and suboptimal temperatures is surprising and merits future investigation.

Pre-activation of *B. japonicum* cells with Ge, JA and MeJA might have caused more infection site initiation, or the success of a greater number of the initiated infections leading to the formation of more nodules (Figure 5.1). Previously, Zhang and Smith (1995) reported that enhanced nodulation of Ge pre-incubated *B. japonicum* cells under low RZT conditions is due to alteration of the time-course of each nodulation stage, but this was especially so for the very earliest steps, probably including signal exchange. Incubation of *B. japonicum* cells with Ge enhanced the early stages of nodule formation thus accelerating the onset of nodule development. Plant-to-bacteria signal molecules have successfully been used to enhance nodulation in several other legume-rhizobia symbioses. For instance the effect of naringenin and hesperatin have been used to enhance pea-*Rhizobium leguminosarum* bv. viceae nodulation (Ahlawat, *et al.,* 1998; Bandyopadhyay *et al.* 1996; Novak *et al.,* 2002; Lira Junior *et al.,* 2003).

5.4.2. Effect of jasmonates on N_2 fixation

Plants receiving pre-incubated *B. japonicum* inoculant fixed more nitrogen than plants receiving the control treatment - *B. japonicum* cells only, without induction (Fig. 5.3). Both JA and MeJA enhanced nitrogen fixation and the effect was concentration dependant at both RZTs. Generally, 50 μM resulted in the greatest increase in fixed nitrogen per plant at both RZTs. However, the Ge effect was temperature dependent. Genisetin at 5 and 10μM increased the nitrogen content of the plants, however further increase in concentration had temperature dependent effects (Fig. 5.3). Twenty μM Ge reduced plant nitrogen content at 25°C RZT while the opposite was true for 17°C. The lower nitrogen content of plants at 25°C, when inoculated with *B. japonicum* cells preinduced with 20 μM genistein, was probably due to the observed formation of fewer and smaller nodules on each plant. Since the seeds used in the experiment were of uniform size, the increases in N content per plant represents differences in the amount of nitrogen fixed. Thus plants receiving rhizobia in which the *nod* genes had been preinduced by the exogenous addition of plant-to-bacteria signals led to the formation of

more nodules with more nodule dry mass, leading to improved N_2 fixation and plant growth as compared to the uninduced control inoculant. This might have resulted in more leaf area (Fig. 5.4) and more leaf chlorophyll production (increased SPAD readings, data not shown), probably leading to increased photosynthesis. An increase in photosynthesis would have led to enhanced dry matter accumulation (Fig. 5.5). The use of inoculant preinduced with plant-to-bacteria signal molecules has previously been shown to increase nodulation and nitrogen fixation of legume crops. For instance, soybean plants inoculated with genistein induced *B. japonicum* had more fixed nitrogen than plants treated with uninduced inoculant. This increase in fixed nitrogen was attributed to more nodules with more nodule dry weight, due to the pre-induction of inoculant with genistein (Zhang and Smith, 1995; Pan *et al.,* 2002).

5.4.3. Effect of jasmonates on plant growth and development

Our results indicate that plants receiving *B. japonicum* preincubated with inducer molecules had increased plant growth and development. Although leaf number was not affected by the inducer molecules (data not shown), at both RZTs inducer molecule treated *B. japonicum* inoculant increased soybean leaf area (Fig. 5.4); both JA and MeJA induced bacterial inoculants caused greater leaf area than the control inoculant, at both RZTs. This effect was concentration dependent. At higher concentrations, both JA and MeJA treatments resulted in increased leaf area (Fig. 5.4). The effect of Ge was temperature dependent. Genistein at higher concentrations increased leaf area at low RZT (17°C) while it reduced leaf area at high RZT (25°C) treatments. The increased leaf area due to inducer molecules may be due to more nitrogen fixation by these plants, since the plants receiving induced bacterial cells had more nodules (Figure 5.1) and nodule dry weight (Figure 5.2).

Plants receiving bacterial cells induced with Ge or jasmonates also accumulated more dry matter than control plants. Both JA and MeJA enhanced plant dry matter accumulation, at both RZTs, with greater effect at higher concentrations. The effect of Ge was temperature dependent; at higher concentrations (20 μM) Ge actually decreased dry matter accumulation at 25°C RZT. However, at low RZT, 20 μM genistein increased

plant dry matter accumulation (Figure 5.5). Our results indicate that the increase in dry matter accumulation due to inducer molecules may be due to enhanced nitrogen fixation (Fig. 5.3) (more nodules with more nodule weight per plant – Figure 5.1 and 5.2) and greater leaf area (Figure 5.4). For this reason, the inducer-treated plants had a growth advantage over control plants, in the form of more photosynthetic activity, leading to more dry matter accumulation (Figure 5.5). The use of flavonoid induced rhizobial inoculants in enhancing nodule initiation and plant growth and development are now well documented in legume crops such as pea (Ahlawat *et al.,* 1998; Bandyopadhyay *et al.* 1996; Lira Junior *et al.,* 2003) and soybean (Zhang and Smith, 1995; Pan *et al.,* 1998; Belkheir *et al.,* 2000). They reported that increases in plant dry matter accumulation, due to inducer molecule additions to inocula, could be the result of increased nodulation and nitrogen fixation by legume plants. Begum *et al.* (2001a,b) also reported that *Rhizobium leguminosarum* bv. viceae induced with flavonoid molecules enhanced pea and lentil dry matter accumulation under greenhouse and field conditions.

A technology based on the use of Ge to induce *B. japonicum nod* genes in order to enhance soybean nodulation and nitrogen fixation has been present in the market place for several years. The commercial product 'Soyasignal', which uses genistein and diadzein has been marketed in North America since 1997 (Zhang and Smith, 2002). Research data collected over 6 years on 'Soyasignal technology', from more than 100 field trials in North America, revealed that this technology improves soybean nodulation and nitrogen fixation and the strongest effects were seen when applied to soils with cool spring temperature conditions (lower than 17°C) (Leibovitch *et al.,* 2001). Although genistein based 'Soyasignal' technology shows promise in enhancing soybean yield, it is exceedingly expensive. It seems that there may now be an opportunity for a similar jasmonate based technology.

5.5. CONCLUSIONS

In conclusion, the results of this study indicated that jasmonates (JA and MeJA) induced *Bradyrhizobium japonicum* inoculants can accelerate nodulation, nitrogen fixation and plant growth of soybean under controlled environment conditions, at both optimal and suboptimal RZT conditions. This is the first report, to the best of our knowledge, of the use of jasmonate signal molecules to manipulate soybean nodulation and nitrogen fixation, leading to increased overall growth. These results thus provide evidence that jasmonates are important signal molecules in *Bradyrhizobium*-soybean symbiosis.

Figure 5.1. **Effect of *B. japonicum* induced with genistein (Ge), jasmonic acid (JA), and methyl jasmonate (MeJA) on nodule number per plant of soybean plants growing at 25 and 17°C RZT. Each value is plotted as the mean ± SE (n = 6)**

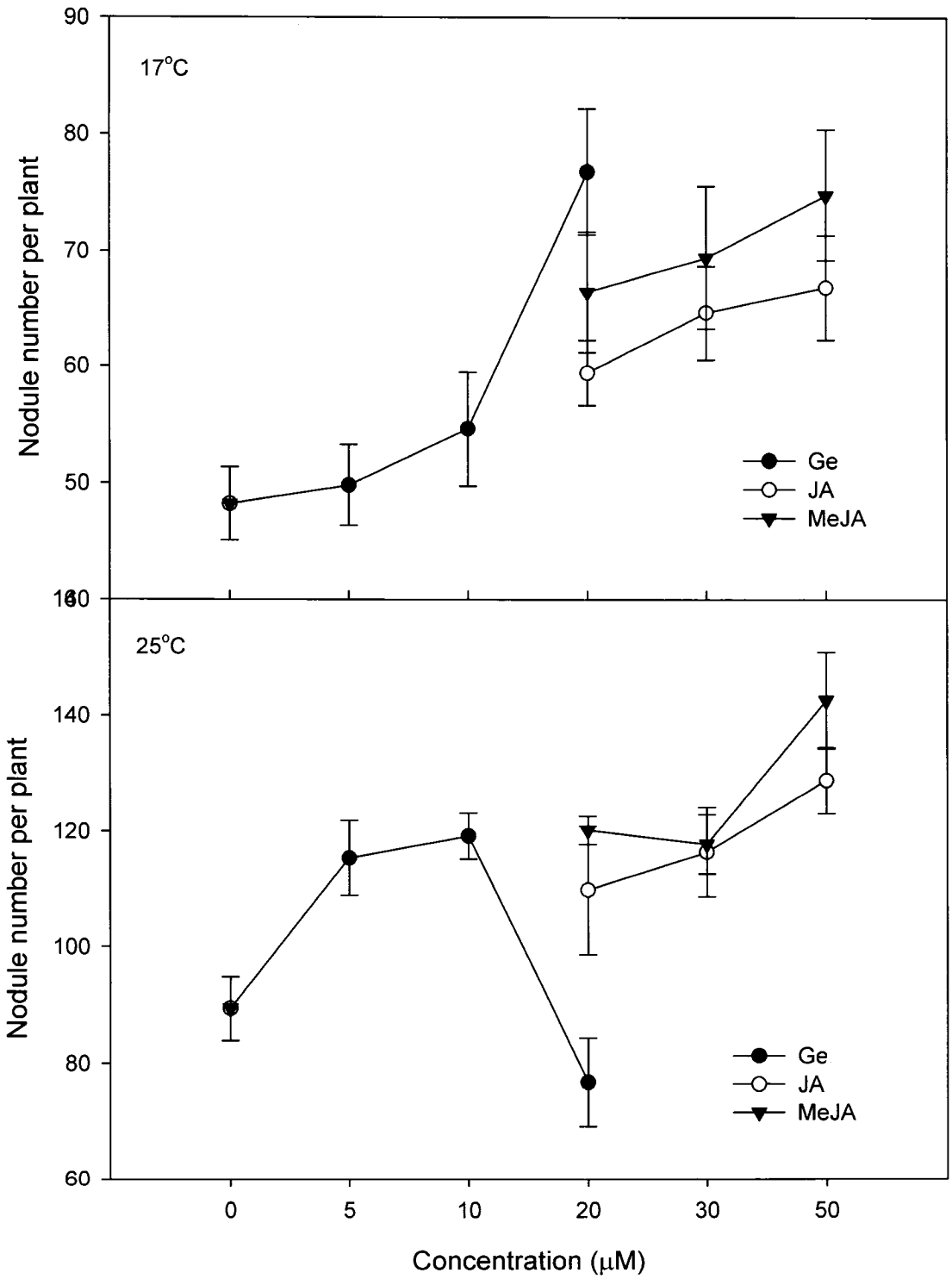

17°C
25°C
Nodule number per plant
Nodule number per plant
90
80
70
60
50
140
120
100
80
60
Ge
JA
MeJA
Ge
JA
MeJA
0
5
10
20
30
50
Concentration (μM)

Figure 5.2. **Effect of *B. japonicum* induced with genistein (Ge), jasmonic acid (JA), and methyl jasmonate (MeJA) on total nodule dry weight (mg) per plant of soybean plants growing at 25 and 17°C RZT. Each value is plotted as the mean ± SE (n = 6)**

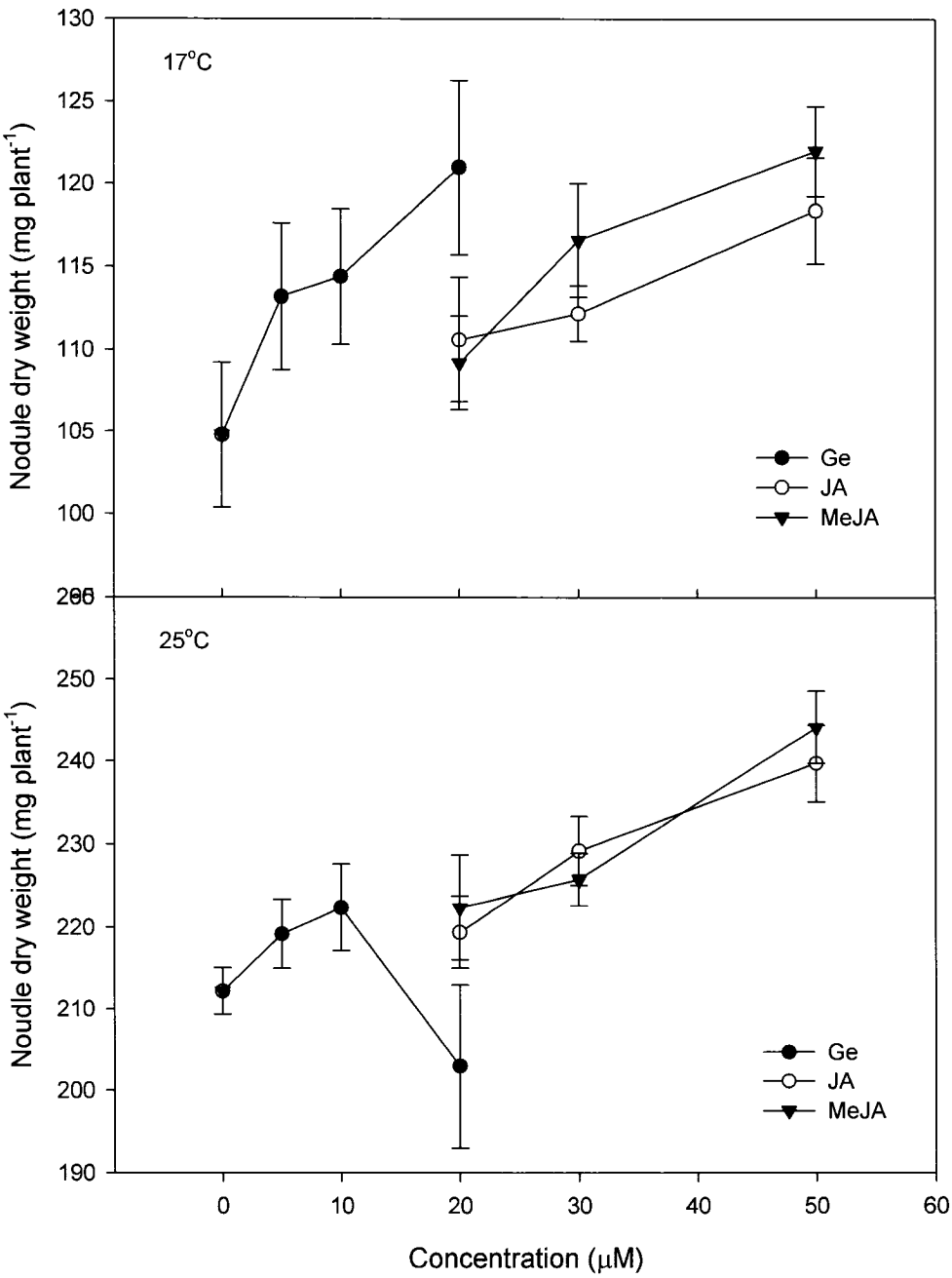

17°C
25°C
Nodule dry weight (mg plant-1)
Noudle dry weight (mg plant-1)
Ge
JA
MeJA
Concentration (μM)
130
125
120
115
110
105
100
250
240
230
220
210
200
190
0
10
20
30
40
50
60

Figure 5.3. **Effect of *B. japonicum* induced with genistein (Ge), jasmonic acid (JA), and methyl jasmonate (MeJA) on total fixed nitrogen (mg) per plant of soybean plants growing at 25 and 17°C RZT. Each value is plotted as the mean ± SE (n = 6)**

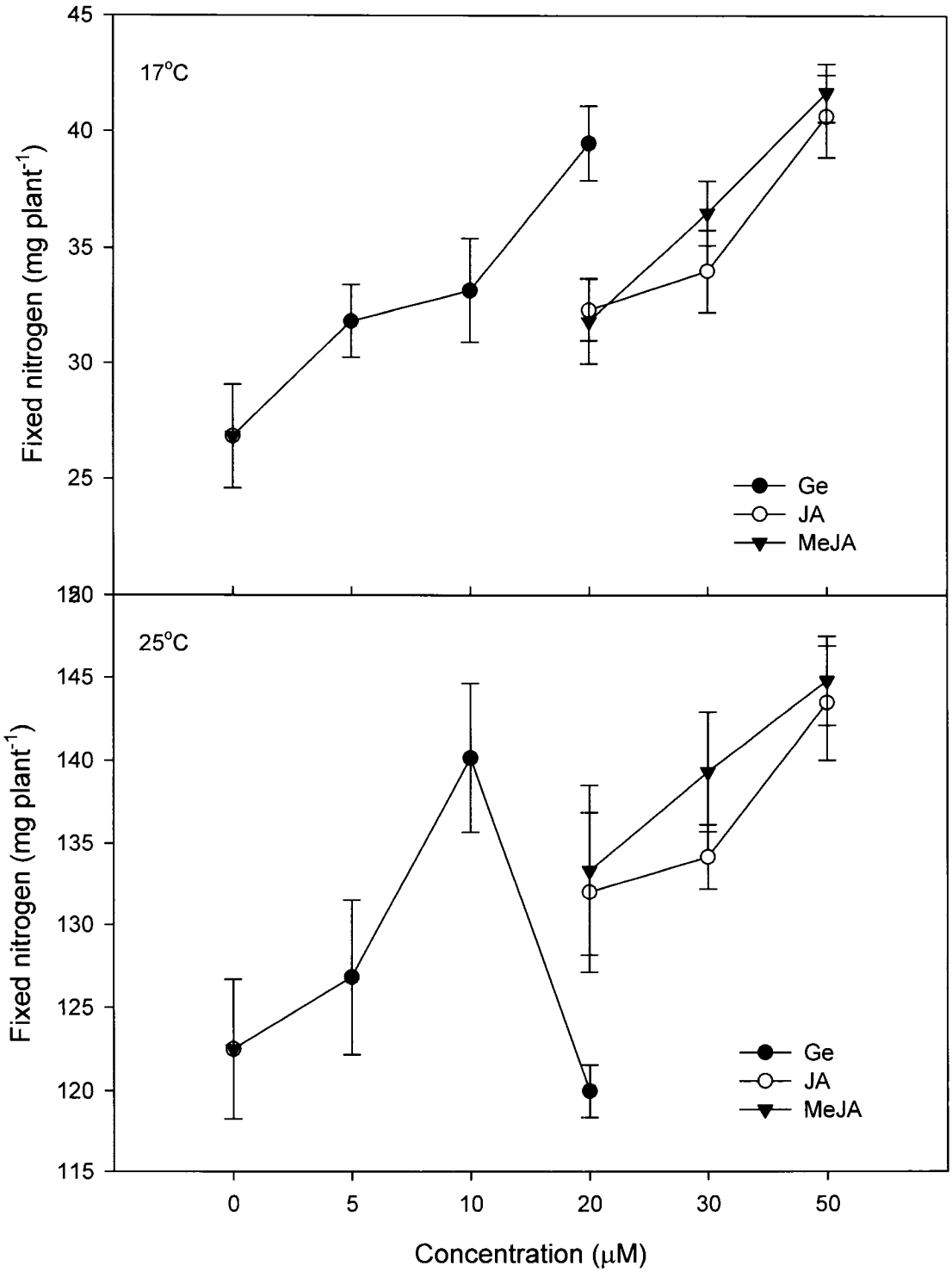

17°C
25°C
Fixed nitrogen (mg plant-1)
Fixed nitrogen (mg plant-1)
Ge
JA
MeJA
Ge
JA
MeJA
45
40
35
30
25
145
140
135
130
125
120
115
0
5
10
20
30
50
Concentration (μM)

Figure 5.4. **Effect of *B. japonicum* induced with genistein (Ge), jasmonic Acid (JA), and methyl jasmonate (MeJA) on leaf area (cm^2) per plant of soybean plants growing at 25 and 17°C RZT. Each value is plotted as the mean ± SE (n = 6)**

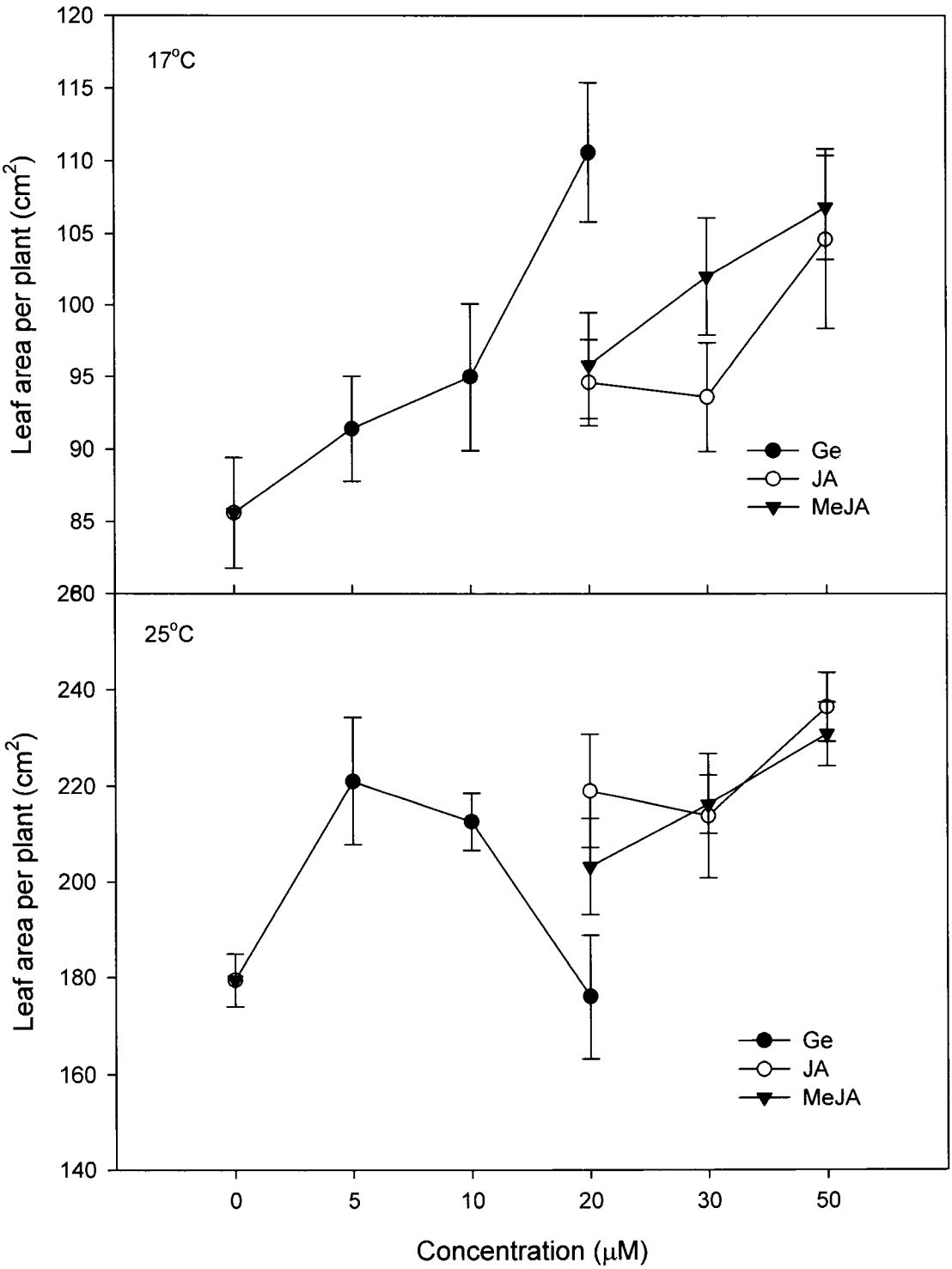
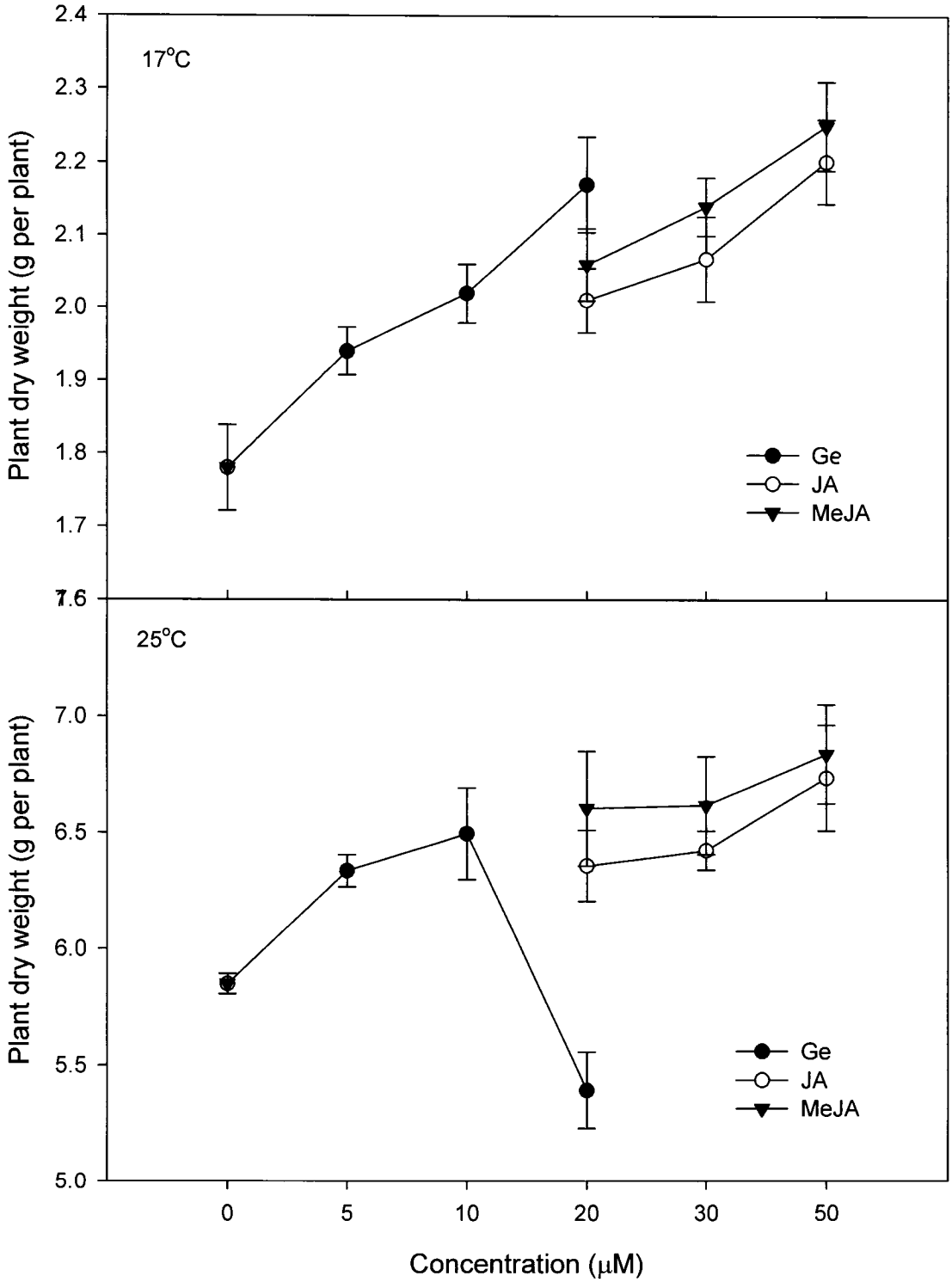

17°C
25°C
Leaf area per plant (cm²)
Leaf area per plant (cm²)
120
115
110
105
100
95
90
85
260
240
220
200
180
160
140
0
5
10
20
30
50
Concentration (μM)
Ge
JA
MeJA
Ge
JA
MeJA

Figure 5.5. **Effect of *B. japonicum* induced with genistein (Ge), jasmonic acid (JA), and methyl jasmonate (MeJA) on dry matter accumulation (g) per plant of soybean plants growing at 25 and 17°C RZT. Each value is plotted as the mean ± SE (n = 6)**


17°C
25°C
Plant dry weight (g per plant)
Plant dry weight (g per plant)
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
7.0
6.5
6.0
5.5
5.0
Ge
JA
MeJA
Ge
JA
MeJA
0
5
10
20
30
50
Concentration (μM)

Preface to Section 6

Section 6 is comprised of a manuscript prepared by myself, Xiaomin Zhou, and Donald L. Smith and will be submitted to *Crop Science* for publication. The format has been changed, as much as possible, to be consistent with the rest of the thesis. All literature cited in this section is listed in the references section at the end of the thesis. Tables and figures are presented at the end of this section.

Low spring soil temperatures in Eastern Canadian soybean production areas hamper, in part, the nodulation process of soybean and this detrimental effect of low temperature on nodulation can be, at least partly, ameliorated by inoculating soybean seeds with genistein pre-treated *B. japonicum* cells (Zhang and Smith, 1996b). Since low temperature can disrupt inter-organismal signaling, genistein induced rhizobia partially overcame this signaling disruption and improved nodulation and nitrogen fixation.

In the previous section (section 5) I demonstrated that *B. japonicum* cells induced with jasmonates (JA and MeJA) can enhance nodulation and nitrogen fixation of soybean plants at both optimal and sub-optimal RZTs under greenhouse conditions. In this section, I conducted experiments to study the role of *B. japonicum* cells induced with MeJA alone or in combination with Ge on soybean nodulation, nitrogen fixation and grain yield under short season field conditions.

Section 6

Pre-incubation of *Bradyrhizobium japonicum* cells with methyl jasmonate (MeJA) alone or in combination with genistein increases soybean (*Glycine max* (L.) Merr.) nodulation, nitrogen fixation and grain yield under short season field conditions

6.1. ABSTRACT

Jasmonates (Jasmonic acid and Methyl Jasmonate) are naturally occurring plant hormones in plants. They are biosynthesized, via the octadecanoid pathway, from its precursor linolenic acid, in response to wounding, biotic and abiotic stresses. Besides their role *in-planta,* jasmonates can also act as signaling molecules in *Bradyrhizobium*-soybean symbioses by inducing the transcription of nodulation genes of *B. japonicum.* Previous studies have shown that soybean nodulation and growth can be enhanced when inoculated with *Bradyrhizobium japonicum* pre-induced with genistein (Ge). Since jasmonates are able to induce nodulation genes of *B. japonicum*, we conducted two field experiments, in southwestern Quebec Canada, in the year 2002 to determine whether pre-incubation of *B. japonicum* with methyl jasmonate (MeJA) alone or in combination with genistein (Ge) prior to inoculation increases soybean nodulation, nitrogen fixation, plant dry matter production and grain yield. At both sites, experiments were designed as randomized complete block design (RCBD) with four blocks. Two *B. japonicum* strains (USDA3 and 532C) and four inducer molecule treatments [control, Ge (20μM), MeJA (50μM), and Ge+MeJA (20μM+50μM)] were used in the study. The bacterial cultures were induced for 24 h with the inducer molecules and then applied into the furrows at the time of planting. Ge and MeJA, alone or in combination, increased nodule number, nodule dry weight, and seasonal nitrogen fixation, compared to the control treatment inoculated with un-induced *B. japonicum.* Genistein and MeJA pre-incubated bacteria also increased plant growth, dry matter accumulation, and grain yield. These results

demonstrate that methyl jasmonate alone or in combination with Ge, can be used to promote soybean nodulation and nitrogen fixation under low spring field temperatures typical of short season soybean production areas.

Key words: *Bradyrhizobium japonicum*, genistein, grain yield, nitrogen fixation, nodulation, jasmonates, soybean (*Glycine max*),

Abbreviations: Ge - genistein; LCOs: Lipo chitooligosaccharides; MeJA: Methyl jasmonate; RCBD: Randomized complete block design; RZT: Root zone temperature

6.2. INTRODUCTION

Leguminous plants have the ability to fix atmospheric dinitrogen through a symbiotic association with bacteria belonging to the genera *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium and Allorhizobium*, collectively called rhizobia. A successful infection leads to the formation of a new plant organ on the roots, and sometimes stems, of the legume host plant, called nodules (reviewed in Spaink, 1996). Rhizobia reside inside the nodules in a differentiated form, the bacteroid, and are able to fix atmospheric dinitrogen into ammonia, which is then utilized by the plant (Hungria and Stacey, 1997).

Soybean [*Glycine max* (L) Merr.] plants can fix 100 to 200 kg ha^{-1} yr^{-1} of atmospheric nitrogen (N_2) while in symbiotic association with *Bradyrhizobium japonicum* (Smith and Hume, 1987). Many environmental factors affect the soybean-*Bradyrhizobium* symbiosis and sub-optimal root zone temperature (RZT) has been shown to negatively affect root nodulation variables. Low temperature affects all stages of nodule formation and function. Studies with soybean plants subjected to suboptimal RZTs at various stages of their life cycle have shown that infection and early nodule developmental processes are the most sensitive stages (Lynch and Smith, 1993; Zhang and Smith, 1994). Low temperature profoundly inhibits early infection processes and nodule initiation by disrupting the inter-organismal signal exchange which occurs at the beginning of the establishment of symbiosis (Zhang and Smith, 1995). Low root zone temperature decreases the biosynthesis and rhizosecretion of genistein from soybean roots into the rhizosphere (Zhang and Smith 1996a). Genistein is an important signal molecule that induces the transcription of nodulation (*nod, noe* and *nol* genes) genes of *B. japonicum* (Kosslak *et al.*, 1987). At the same time, low temperatures inhibit the transcription of nodulation genes in *B. japonicum* (Zhang *et al.*, 1996) demonstrating that low temperatures negatively affect both signals (plant-to-bacteria and bacteria-to-plant), both of which are crucial for the establishment of nodulation.

McKay and Djordjevic (1993) observed that Nod factor production, and particularly secretion, from bacterial cells was restricted by a range of environmental conditions associated with poor nodulation under field conditions. At 28°C bacterial cells

produced ample Nod metabolites, however, a sharp reduction in Nod metabolite production was observed when the temperature was reduced to 18°C (McKay and Djordjevic, 1993). Recently Zhang *et al.* (2002) have also shown that the ability of *B. japonicum* to produce Nod factors decreases at suboptimal growth temperatures. Besides detrimental effects of low RZT on the early signal exchange process of the two symbionts, it also inhibits the nitrogen fixation by already established nodules, and this inhibition is, at least in part, associated with nodule oxygen permeability (Walsh and Layzell, 1986).

Jasmonic acid and its derivatives, collectively known as jasmonates, are involved in plant responses to biotic and abiotic stresses, as well as plant development (Creelman and Mullet, 1997; Wasternack and Parthier, 1997). Jasmonates play a central role in the wound induced complex of signal transduction pathways in plants (Ryan, 2000). The wounding of plants is associated with an increase in the endogenous levels jasmonates which act as signals and induce genes involved in plant defense responses (Creelman and Mullet, 1997; Farmer *et al.,* 1998; Ryan, 2000). Regulation of JA activation in plants is poorly understood, however, elevated levels of JA are usually correlated with the activation of genes coding for enzymes of the JA biosynthetic pathway (reviewed by Wasternack and Hause, 2002). The biosynthesis of JA starts with linoleic and linolenic acid which is produced from membrane lipid breakdown though the action of phospholipase. These fatty acids are converted into 13-hydroperoxylinolenic acid, which is then converted into 12, 13 epoxy-octadecatrienoic acid which is then catalyzed into 12-oxo-phytodienoic acid which, following reduction and three steps of ß-oxidation, produces (+)-7-iso-JA (Creelman, and Mullet, 1997, Vick and Zimmerman, 1984).

We have shown that, besides its roles in-planta, JA and MeJA can also act as important signaling molecules in the *Bradyrhizobium*-soybean symbiosis. We have recently shown that jasmonates induce Nod factor production from *B. japonicum* (Section 3 of this thesis). *Bradyrhizobium japonicum* cells induced with JA or MeJA also produced more LCO (Section 3 of this thesis) suggesting a potentially important role for these compounds in the *Bradyrhizobium*-soybean symbiosis.

Since Ge pre-incubation of *B. japonicum* partially overcomes the detrimental effects of low RZT and accelerates nodule development and the onset of nitrogen

fixation, it is now used in some commercial inoculants as a way to increase soybean yield, especially under sub-optimal soil temperature conditions. Genistein is commercially available, however, it is expensive and reducing the cost of commercial inoculant production could prove vital both for industrial inoculant producers and the end users. In addition, incubation of *B. japonicum* cells with the higher concentrations of Ge required to overcome low RZT effects, inhibits the growth of *B. japonicum* cultures. Pre-incubation of inoculants with MeJA is an alternative; it is an effective inducer of *nod* genes of *B. japonicum* (Section 3 of this thesis), is substantially cheaper than genistein and does not have negative effects on *B. japonicum* cells. In addition, MeJA promotes lateral root initiation and growth at lower concentrations (less than 10^{-8} M - Tung et al., 1996) demonstrating its role in promoting root growth at lower concentrations. In this study we report that MeJA induced *B. japonicum* inocula improve soybean yield under short season field conditions.

6.3. MATERIALS AND METHODS

6.3.1. Experimental design

The experiment was structured as a Randomized Complete Block Design (RCBD) with four replicates. The treatments were the result of factorial combinations of two factors (inducer molecules and bradyrhizobial strains). The inducer molecule treatments were: 1) *B. japonicum* culture pre-incubated with 20 μM genistein, 2) *B. japonicum* culture pre-incubated with 50 μM MeJA, 3) *B. japonicum* culture pre-incubated with both 20 μM genistein and 50 μM MeJA, and 4) *B. japonicum* culture without any inducer molecule(s) (un-induced inoculant control). In order to determine the amount of nitrogen fixed by the *B. japonicum*-soybean symbiosis when the rhizobia have been activated with different inducer molecules, non-nodulating Evans line were also included in the experiment. Each block had one plot of non-nodulating Evans line randomly assigned. The two bradyrhizobial strains used in the experiment were 532C (Hume and Shelp, 1990) and USDA3. Strain 532C was used since this strain performs well under cool Canadian soil conditions, is included in commercial inoculants used in Eastern Canada (Hume and Shelp, 1990) and because it demonstrated superior performance over a range of temperatures (Lynch and Smith, 1993). USDA3 was selected due to its enhanced sensitivity to inducer molecules (Section 3 of this thesis).

6.3.2. Field conditions and plant material

Two experiments were conducted at the Emile A. Lods Research Center (45° 25' 45'' N latitude and 73° 56' 00'' longitude) of the Macdonald Campus of McGill University, Montreal, Canada in the year 2002. The experiment was performed at two sites. One site was on a sandy-loam soil (fine silty, mixed, nonacid, frigid Humaquept) where barley had been grown in the previous year. The second site was on a clay-loam soil (fine, mixed, nonacid, frigid Humaquept) where corn had been grown in the previous year. At both sites, the soybean cultivar OAC Bayfield was sown.

Each plot was 5 x 1.4 m (row length of 5 m and width of 0.20 m with 7 rows in each plot). Soybean seeds were sown by hand and the plant population was 350 plants per plot (450,000 plants ha^{-1}) with an approximate plant-to-plant distance of 10 cm within the row. Soybean seeds were sown on May 21 and May 25, 2002 at the clay-loam site and sandy-loam site, respectively.

6.3.3. Bacterial inoculum preparation

B. japonicum strains 532C and USDA3 were cultured from Petri plate colonies in 500 mL flasks containing 100 to 200 mL yeast extract mannitol (YEM) culture medium (Vincent, 1970). The cultures were shaken at 150 rpm at 28°C. The initial culture time was 7 days, which was followed by subculturing for a further 5 days in 4 L flasks containing 2 L of bacterial culture. Filter sterilized Ge and MeJA (Sigma-Aldrich, Mississauga, ON, Canada) stock solutions were prepared in dimethyl sulfoxide and added to the subcultures so that the final Ge and MeJA concentrations of the bacterial cultures were 20 and 50 μM, respectively. The control flasks of the two strains were not induced. The sub-cultures were shaken for another 24 h as described previously. Before inoculation of the seeds in the field, the cell density of the subculture was determined by spectrophotometer at 620 nm and diluted with distilled water to an O.D. of 0.1 [(A_{620} nm reading of 0.1 indicates approximately 10^8 cells/mL (Bhuvaneswari *et al.*, 1980)].

6.3.4. Inoculation and planting

Soybean seeds were sown into open rows and covered with soil immediately after the inoculants were added. The inoculant was applied to the seeds in the open furrows using a 60 mL sterile plastic syringe. A 60 mL inoculant dose was applied evenly to each row and the row was then closed immediately. No inoculant was applied to the non-nodulating plots. In order to achieve the desired stand of 450,000 plants ha^{-1}, seedlings were thinned after emergence.

6.3.5. Data collection

Daily average air temperature and precipitation were recorded at the Macdonald Campus weather station of McGill University, Ste-Anne-de-Bellevue, Quebec, Canada during the course of experiment and are shown in Figure 6.1. The classification of soybean growth stages followed those of Fehr *et al.* (1971). Plants were harvested at three developmental stages during the season: (i) V3, three nodes on the main stem with fully developed leaves beginning with the unifoliate nodes (July 7, 2002), (ii) R3, at the early pod development stage (August 12, 2002) and (iii) R8, harvest maturity stage (September 28, 2002). At each harvest plant samples (10 plants randomly selected) were harvested from each plot for data collection. During the first two harvests, plant samples were separated into shoots and roots and the roots washed with tap water. Nodules were removed from roots and the number of nodules per plant was recorded. The nodules were then oven dried at 70 °C for at least 48 h and nodule dry weight (mg) per plant was recorded. At each harvest, shoots and roots were oven dried at 70 °C for at least 48 h and the nitrogen concentration was determined separately using an Elemental Analyzer (NC2500 Elementary Analyzer, Thermoquest Italic, S.P.A. Italy). At the end of the growing season, R8 - harvest maturity stage, the plants were harvested for yield component determination. Ten plants were randomly selected from each plot, harvested and the number of pods and plant height were recorded. The plants were then oven dried at 70°C for at least 48 h and weighed. The seeds were threshed by hand and total seed yield per plant and 100-seed weight were determined. The seed nitrogen concentration was determined using an automated elemental analyzer (Model NC 2500; CE Instruments, Milan, Italy). Samples from the non-nodulating Evans line were also collected and were used for detecting the differences in amount of nitrogen fixed by various *B. japonicum* treatments by nitrogen difference method. The total amount of seed nitrogen fixed was calculated by nitrogen difference method (Total seed N fixed = Total N in seeds of nodulating plants – Total N in seeds of non-nodulating plants). Seed N is a suitable way to assess effects of various treatments on total N fixation as the nitrogen harvest index (proportion of plant N in the seeds) is generally about 0.8 (Smith *et al.*, 1988). Relative leaf chlorophyll content was measured by taking SPAD (Soil Plant Analysis Device)

meter readings at two different stages (V3 and R3) of the plant growth. In order to calculate yield per plot, a plot area of 2.8 m^2 (2 m length x 1.4 m width) was harvested with a small plot combine harvester (Winterstieger, Salt Lake City, UT). There were 7 rows in each plot and the harvested segment of each row was 2 m long. The seeds were oven dried at 70°C for at least 48 h and weighed. The yield data per plot was extrapolated to yield ha^{-1}.

6.3.6. Data analysis

The data collected were analyzed by analysis of variance (ANOVA) using the statistical analysis system (SAS) computer package. Means were compared by orthogonal contrasts (Steel and Torrie, 1980) in order to test responses to inducer molecules [contrast 1: comparison between controls (532C control + USDA3 control) and the inducer molecule treatments applied to both strains {Ge+MeJA+(Ge+MeJA)}], between strains (contrast 2: comparison between strain 532C and USDA3), between control and the inducer molecules applied to strain 532C [contrast 3: comparison between control and {Ge+MeJA+(Ge+MeJA)}] and between control and the inducer molecules applied to strain USDA3 [contrast 4: comparison between control and {Ge+MeJA+(Ge+MeJA)}]. Differences were declared significant at $P<0.05$. Linear regression procedures of SAS were used to determine the relationships between variables.

6.4. RESULTS AND DISCUSSION

The daily temperature and precipitation during the 2002 growing season (May – September) are shown in Figure 6.1, while the average monthly temperature and monthly precipitation for the past 57 years (1945-2002) in shown in Figure 6.2. The first two months of 2002 were cooler and wetter, while the remaining three months were hotter and drier, than the 57 years average (Figure 6.2). The average temperature at planting (11.3 ^{o}C, May) was 1.8 ^{o}C below the 57 years average (13.1 ^{o}C) while the average precipitation in the month of May (128.5 mm) was much higher (55.9 mm, or 77%) than the 57 year average (72.6 mm). During early plant growth and development (June), the conditions were wetter and cooler than the long-term average (Figure 6.2).

6.4.1. Nodulation

The results of our study indicated that Ge, MeJA and both inducer molecules together consistently increased nodule number and nodule dry weight over the untreated control (inoculated with un-induced *B. japonicum* only) at both sites (Table 6.1). In general plants receiving both inducers together produced more nodules with more dry weight (Table 6.1).

At the sandy loam site, plants inoculated with strain 532C and treated with inducers had 60.7% (V3 stage) and 10.8% (R3 stage) more nodules than the uninduced control; there were 83.6% (V3 stage) and 25.2% (R3 stage) increases over control in the case of USDA3. Nodule dry weight increased by 28.1% (V3 stage) and 9.2% (R3 stage) over the non-induced control with strain 532C while the increase was 36.0% (V3 stage) and 8.2% (R3 stage) for induced USDA3 as compared to the non-induced control. At the clay-loam site, plants inoculated with induced strain 532C had 72.5% (V3 stage) and 44.7% (R3 stage) more nodules than the uninduced control, while the increase was 85.2% (V3 stage) and 60.4% (R3 stage) over the control in the case of USDA3. The nodule dry weight increased by 59.4% (V3 stage) and 22.9% (R3 stage) over control with strain 532C while the increases was 106.2% (V3 stage) and 14.6% (R3 stage) when using preincubated USDA3 with inducers, as compared to uninduced control (Table 6.1).

There was often no difference ($P < 0.05$) between the two tested strains, 532C and USDA3, for nodule number and nodule dry weight per plant at either harvest., The exceptions were nodule dry weight at the V3 stage (sandy-loam site) and nodule dry weight at the R3 stage (clay-loam site) where inoculation with strain 532C resulted in 8.6% and 10.7% increases in nodule dry weight over USDA3 at the sandy-loam (V3) and clay-loam sites (R3), respectively (Table 6.1). The superior performance of strain 532C under low spring soil Canadian conditions has already been documented (Hume and Shelp, 1990).

Our finding that Ge increased nodulation is consistent with previously published results (Pan *et al.* 2002, 1998; Zhang and Smith, 1997, 1995; Belkheir *et al.*, 2000). Earlier publications found increased nodule number and weight following preincubation of *Bradyrhizobium japonicum* with Ge. Our data indicate that inoculation of soybean plants with bradyrhizobial cells incubated with MeJA alone or in combination with Ge enhanced soybean nodule number and weight. The mechanism by which MeJA enhances soybean nodulation is probably similar to that of Ge. *B. japonicum* incubated with Ge (Kosslak *et al.*, 1987) and MeJA (Section 3 of this thesis) promote the expression of *nod* genes, leading to higher levels of Nod factor production by *B. japonicum* cells. The *nod* genes of *B. japonicum* cells were more effectively induced when the cultures were treated with Ge and MeJA together than with either alone (Section 3 of this thesis) suggesting a different induction mechanism for these two inducer compounds. Soybean plants inoculated with *B. japonicum* cells that had been pre-incubated with either Ge, MeJA or both together formed nodules earlier and faster than plants treated with non-induced inoculant. This is the first report that MeJA induced *B. japonicum* increased nodule number and nodule weight under field conditions. We have previously shown, under controlled environment conditions, that MeJA preincubated *B. japonicum* increased nodule number and weight under both optimal and suboptimal RZT conditions (Chapter 5 of this thesis)

Low temperatures inhibit genistein biosynthesis and rhizosecretion from soybean roots (Zhang and Smith, 1996a). They also inhibit the expression of *nod* genes by *B. japonicum* (Zhang et al., 1996), thus reducing the production of Nod factors (LCOs) (McKay and Djordjevic 1993; Zhang *et al.*, 2002; Duzan, 2004). Previous studies have

shown that genistein preincubation of *Bradyrhizobium* helps overcome low soil temperature inhibition of soybean nodulation (Zhang and Smith, 1995). Since the spring and early summer of 2002 were cooler than the long term average (Figure 6.2), the preincubation of bacterial cells with Ge, MeJA, or both inducers together, might have been particularly effective in increasing soybean nodule number and weight.

Nodule number and dry weight were different between plants at the two sites (Table 6.1). Plants growing at the sandy-loam site had more nodules and more nodule dry weight at both harvests than plants growing at the clay-loam site (Table 6.1) indicating that differences in soil type and/or planting date resulted in quite different plant nodulation responses. Physical and chemical properties of soil are known to affect both nodule formation and subsequent nodule growth (Gibson, 1971).

6.4.2. Plant growth and biomass production

Contrast analysis of the data showed that inducer treatments increased leaf area per plant ($P < 0.05$) at both harvests at the sandy-loam site. However, there was no effect ($P < 0.05$) of inducer treatments on leaf area at the R3 developmental stage at the clay-loam site, while the contrast analysis showed a difference ($P < 0.05$) at the V3 stage (Table .6.2). There was no difference between the two strains for leaf area at the V3 stage at the sandy-loam site, while there was a difference ($P < 0.05$) at the R3 developmental stage. At the clay-loam site, there was no difference ($P < 0.05$) between the two strains for leaf area (Table 6.2)

A contrast analysis of all inducer treatments versus the inoculant only control showed increases in dry matter accumulation ($P < 0.05$) due to inducer treatments on plant dry matter accumulation at both harvests at both sites (Table 6.2). However, there was no difference between the two strains ($P < 0.05$) for plant dry matter accumulation at either harvest at both sites. The increased dry matter accumulation due to the application of *B. japonicum* induced with Ge, MeJA or both together is probably related to enhanced nodule number and nodule weight (Table 6.1) which would have led to increased overall nitrogen fixation, leading to more photosynthesis and dry matter accumulation. The use of genistein to increase soybean dry matter accumulation has previously been

documented (Zhang and Smith, 1995; 1997) and our results agree with those findings. Pan *et al.,* (1998; 2002) also reported that *B. japonicum* induced with Ge promotes dry matter accumulation by soybean plants. Our finding that MeJA pre-incubated *B. japonicum* inocula increased plant biomass and dry matter accumulation expand these earlier findings to include jasmonate inducers. We have shown that treatment of *B. japonicum* cells with MeJA prior to inoculation of soybean promotes soybean dry matter accumulation under controlled environment conditions under both optimal and suboptimal conditions (Chapter 5 of this thesis).

There were differences in plant growth and dry matter accumulation between the two sites (Table 6.2). Plants growing at the sandy-loam site accumulated more dry matter than plants at the clay-loam site. Plants at the sandy-loam site formed more nodules, had greater nodule dry weight values and (Table 6.1) and fixed more nitrogen, and these factors probably caused the greater dry matter than at the clay-loam site.

6.4.3. Shoot and seed nitrogen yield and total nitrogen fixed

At R3 developmental stage, there were differences ($P < 0.05$) among inducer treatments for shoot nitrogen yield at both sites. Preincubation of *B. japonicum* with Genistein, MeJA and both inducers together increased shoot nitrogen yield at both sandy and clay loam sites, as compared to the uninduced control treatment (receiving *B. japonicum* inoculant only) (Table 6.3). At the clay-loam site, preincubation of *B. japonicum* strain 532C with Ge, MeJA or both inducers together resulted in 11.8, 19.8 and 10.1% increases, respectively, for shoot nitrogen yield over the control treatment, while preincubation of strain USDA3 with Ge, MeJA or both inducers together caused 18.1, 9.0 and 23.3% increases, respectively, for shoot nitrogen yield over the control. At the sandy-loam site, strain 532C preincubated with Ge, MeJA or both inducers together resulted in 9.5, 12.8 and 12.8% increases, respectively, for shoot nitrogen yield, while for strain USDA3 the corresponding figures were 7.9, 5.9 and 16.0%, respectively (Table 6.3). Given that the amount of nitrogen extracted from the soil should be similar for both control and inducer treatments, the differences between the two amount to difference in

total amount of nitrogen fixed. There was no difference ($P < 0.05$) in shoot nitrogen yield between the two tested strains at both sites (Table 6.3).

Shoot fixed nitrogen was also affected ($P < 0.05$) by inducer treatments at both sites. At the clay-loam site, preincubation of *B. japonicum* strain 532C with Ge, MeJA or both inducers together resulted in 10.9, 19.6 and 12.2% increases, respectively, for shoot nitrogen fixed over the control treatment, while preincubation of strain USDA3 with Ge, MeJA or both inducers together caused 18.2, 11.8 and 23.1% increases, respectively, for shoot nitrogen fixed, over the control. At the sandy-loam site, strain 532C preincubated with Ge, MeJA or both inducers together resulted in 7.5, 6.1 and 8.1% increases, respectively for shoot nitrogen fixed, while for strain USDA3 these figures were 9.4, 10.5 and 20.1%, respectively (Table 6.4). The increased amounts of nitrogen fixed by plants receiving bacterial cultures induced with Ge, MeJA or both inducers together was related to increases in nodule number and nodule dry matter per plant (Table 6.4). At both sites, the amount of seed nitrogen fixed was related to nodule number per plant ($R^2 = 0.6461$, $P < 0.05$, at the sandy-loam site; $R^2 = 0.6308$, $P < 0.05$, at the clay-loam site), and nodule dry weight per plant ($R^2 = 0.7614$, $P < 0.05$, at the sandy-loam site; $R^2 = 0.5606$, $P < 0.05$, at the clay-loam site). There was no difference ($P < 0.05$) in shoot nitrogen fixed between the two tested strains at both sites (Table 6.4).

Our data also showed that site had a large effect on the ability of soybean plants to fix nitrogen. Plants growing at the sandy-loam site fixed more nitrogen than plants growing at the clay-loam site (Table 6.3, 6.4). The superior performance of plants at the sandy-loam soil was probably due, at least for the most part, to the greater nodule number and dry weight (Table 6.1) at this site, as compared to the clay-loam site.

6.4.4. Plant height

The application of genistein, methyl jasmonate, or both inducers together did not affect ($P < 0.05$) plant height (at harvest maturity, R8 stage) at either site. This is in general agreement with the findings of Zhang and Smith (1996b) and Pan *et al.* (1998), who reported that inducer molecules had little effect on plant height. Contrast analysis of all inducer molecule treatments versus the inoculant only control also did not detect any

differences in plant height due to the applied treatments. There was no difference in plant height between the two tested strains.

Plants growing at the sandy-loam site were taller (Table 6.5), less branched and had shorter branches (data not shown) than those growing at the clay-loam site (Table 6.6), indicating that the combination of soil type and plant date differences resulted in quite different growth environments. The differences between the two sites (physical and chemical properties of soil plus planting date) affected both plant growth and development. Belkheir *et al.* (2001) reported that plant height was more sensitive to weather and soil conditions than inducer molecule effects.

6.4.5. Seed nitrogen yield and nitrogen fixed

Inducer molecule treatments increased ($P < 0.05$) seed nitrogen yield and seed nitrogen fixed at both sites. There was no difference between the two tested strains for seed nitrogen yield and seed nitrogen fixed at both sites (Table 6.4). At both sites, the highest seed nitrogen yield and seed nitrogen fixed were obtained for the plants receiving inoculants induced with Ge+MeJA. The control plants had the lowest nitrogen yield. At the sandy-loam site, incubation of *B. japonicum* strain 532C with Ge resulted in 12.37%, MeJA in 13.42% and both inducers together in 37% increases in seed nitrogen fixed over control, while incubation of strain USDA3 with Ge resulted in 8.4%, MeJA in 6.6% and both inducers together in 10.78% more than the control. At the clay-loam site, incubation of *B. japonicum* strain 532C with Ge resulted in 9.19%, MeJA in 10.73% and both inducers together in 11.61% more seed fixed nitrogen than the control, while incubation of strain USDA3 with Ge resulted in 9.53%, MeJA in 10.89% and both inducers together in 14.35% more than the control.

The increase in nitrogen yield due to Ge treatment corroborates previous findings (Belkheir *et al.*, 2001; Pan *et al.*, 1998; Zhang and Smith, 1996b). Since the mechanism of action of MeJA is almost certainly similar to that of Ge, we speculate that MeJA induced increases in seed nitrogen yield are due to a more rapid nodulation and onset of nitrogen fixation, as previously reported with Ge. When bacterial cells were induced simultaneously with Ge and MeJA, there was some synergy between these inducers for

seed nitrogen yield. These findings, and especially the increases in N in plant tissues, argue strongly that the effect of Ge and MeJA inducers followed from an earlier onset of N_2 fixation and increased total fixation.

Site had a large effect on the soybean seed nitrogen yield. Plants growing at the sandy-loam site had greater seed nitrogen yields and nitrogen fixed than those growing at the clay-loam site (Table 6.4). The increased seed nitrogen yield and seed nitrogen fixed by plants growing at the sandy-loam site was probably related to nodule number and nodule dry weight (Table 6.1) per plant. At both sites, there was a relationship between nodule number per plant ($R^2 = 0.4440$, $P < 0.05$, at the sandy-loam site; $R^2 = 0.4775$, $P < 0.05$, at the clay-loam site), nodule dry weight per plant ($R^2 = 0.6996$, $P < 0.05$, at the sandy-loam site; $R^2 = 0.7454$, $P < 0.05$, at the clay-loam site) and the amount of seed nitrogen fixed. The amount of seed nitrogen fixed was also correlated with grain yield at both sites ($R^2 = 0.6257$, $P < 0.05$ at the sandy-loam site; $R^2 = 0.6424$, $P < 0.05$, at clay-loam site).

6.4.7. Grain yield

Plants receiving *B. japonicum* induced with Ge (20μM), MeJA (50μM) or both compounds together (Ge 20μM + MeJA 50μM) had greater grain yields than the control plants (receiving uninduced cultures) at both sites. A contrast analysis of all inducer molecule treatments versus the inoculant only control showed increases ($P < 0.05$) due to inducer treatments for soybean grain yield at both harvests at both sites (Tables 6.5, 6.6). At the sandy-loam site, when inoculated with *B. japonicum* strain 532C, soybean yields were 10.9% higher when pre-treated with Ge (20μM), 17.4% with MeJA (50μM) and 16.3% with Ge+MeJA, over control plants receiving *B. japonicum* only as inoculant. When inoculated with *B. japonicum* strain USDA3 soybean yields were 6.4% higher when pretreated with Ge (20μM), 7.4% with MeJA (50μM) and 17.0% with Ge+MeJA (Table 6.5). At the clay-loam site, application of preinduced *B. japonicum* strain 532C resulted in yield increases of 8.6% with Ge (20μM), 7.4% with MeJA (50μM) and 8.6% with Ge+MeJA, over control plants receiving *B. japonicum* only as inoculant; while these

figures were 14% for Ge (20μM), 15.4% with MeJA (50μM) and 14.5% with Ge+MeJA for strain USDA3 (Table 6.6).

At the sandy-loam site there was no difference between the two tested strains for soybean yield, while there were differences between the two inoculant strains ($P < 0.05$) for soybean yield at the clay-loam site, with 532C inoculant resulting in yields 9% higher than USDA3. The superior performance of strain 532C under short season conditions has been previously reported (Hume and Shelp, 1990).

A stimulative effect of Ge induced inoculants on soybean grain yield has previously been reported (Zhang and Smith, 1996b: Pan *et al.*, 1998; Belkheir *et al.*, 2001) and our results are in agreement with those findings. However, the increase in grain yield due to genistein in our experiment was lower than the previously published results (Zhang and Smith 1996b; Pan *et al.* 1998; Belkheir *et al.* 2001). The smaller increase in yield in our experiment may have been due to the hotter and drier conditions during the reproductive stage of the soybean plants, and especially during pod filling (Figure 6.2).

B. japonicum cells induced with MeJA and inoculated onto soybean plants resulted in increased grain yield (Tables 6.5, 6.6). There is no previous report of soybean grain yield increase using MeJA alone or in combination with Ge to promote soybean yield. The mechanism underlying an increase in soybean grain yield by MeJA induced inocula, as compared to control treatments, is probably similar to that of Ge. Both Ge and MeJA induce the expression of nodulation genes leading to the biosynthesis of Nod factor production (Section 3 of this thesis) by *B. japonicum*. However, the finding that Ge+MeJA resulted in greater grain yield, at least at the sandy-loam site, than either MeJA or Ge alone, suggest that the synergistic effect of MeJA plus Ge on soybean yield might be due to enhanced nodulation gene expression in the presence of both inducer molecules (Section 3 of this thesis). Increased nodulation gene expression and Nod factor production could accelerate the onset of N_2 fixation, leading to an earlier onset of growth not limited by N supply, or the Nod factors could stimulate growth directly (Prithiviraj *et al.* 2003).

There was variation in grain yield between the two soil types. Plants growing in sandy –loam soil produced greater grain yields than those growing in clay-loam soil. The

collected data suggest that the greater grain yield in the sandy-loam site was due to vigorous plant growth and efficient nodulation as compared to the clay-loam site (Tables 6.1 and 6.2). The unusually cool and wet conditions experienced at the beginning of the growing season would have more adversely affected soybean growth on a heavier soil than a lighter one as clay soils retain more water leading to lower soil temperatures and slower germination and seedling growth. The early spring conditions in 2002 were very wet and cool in the months of May and June, while much of the subsequent growing season was hot and dry (Figure 6.2). The soil at the clay-loam site was heavy clay to at least 1 m of depth. The soil at the sandy-loam site was sandy-loam to a depth of almost 1 m, underlain by clay. At the clay-loam site, the cool wet conditions slowed the early development of the seedlings, relative to development at the sandy-loam site, and, as the soils rapidly dried out these plants were probably left with a less deep root system (data not shown) and was probably more affected by the subsequent dry conditions than the plants at the sandy-loam site. Plants at the sandy-loam site established better and were always bigger than those at the clay-loam site (Table 6.2) and subsequently yielded more (Tables 6.5 and 6.6). Roots of the plants at the sandy-loam site were generally bigger (visual observation) and probably deeper, establishing well in the lighter soil during the cool and wet spring, and reaching deeper into the soil profile, allowing them to access more soil moisture from the deeper clay layer through the mid season dry period. In spite of the large differences in the pattern of development between the two sites, we observed a similar pattern of effects following inoculation of soybean plants with *B. japonicum* cells treated with inducer compounds. The findings appear to be consistent across a range of environmental conditions, and therefore quite robust.

6.4.8. Yield components

A contrast analysis of all inducer molecule treatments versus the inoculant only control showed increases ($P < 0.05$) due to inducer treatments for pod number per plant and seeds per plant at both sites. *B. japonicum* induced with Ge+MeJA produced the maximum number of pods per plant, followed by Ge and then MeJA (Tables 6.5, 6.6). In general, all the inducer molecules increased pod number per plant and seeds per plant relative to the

B. japonicum alone inoculant. Seed number per pod was not different among treatments at both sites (Tables 6.5, 6.6). A contrast analysis of the two tested strains did not show any difference between them ($P < 0.05$) for pods per plant, seeds per plant or seeds per pod.

There were differences ($P < 0.05$) among inducer treatments for 100-seed weight. At both sites, maximum 100-seed weight occurred for plants receiving MeJA followed by Ge+MeJA. In general, *B. japonicum* treated with inducer molecules increased 100-seed weight as compared to inoculation with uninduced *B. japonicum* cells. The increased 100-seed weight for plants inoculated with induced *B. japonicum* cells is in agreement with some previously published results (Belkheir *et al.*, 2001) but contrasts with others (Zhang and Smith 1997). The latter showed no effect of genistein inoculant induction on 100-seed weight, but did demonstrate increase in seed number per plant. Our results show that MeJA also increases 100-seed weight and we assume that the mechanism underlying an increase in soybean seed weight by MeJA, as compared to control plants, is similar to that of Ge. Both Ge and MeJA induce Nod factor production (Section 3 of this thesis) from induced bacterial cultures, and thus MeJA induced cultures might have enhanced soybean seed weight by accelerating the establishment of nodules and nitrogen fixation (Cite some tables here), or through growth stimulation caused by Nod factor (Souleimanov *et al.*, 2002). There was no difference ($P < 0.05$) between the two tested strains for 100-seed weight at either site (Tables 6.5, 6.6).

Pod number per plant, seed number per pod, and seed number per plant were not affected by site. The effect of site was pronounced for 100-seed weight. Plants at the sandy-loam site had greater 100-seed weights than plants at the clay-loam site. This greater 100-seed weight seems to have been the main determinant of the higher grain yields at sandy-loam site, as compared to the clay-loam site (Tables 6.5, 6.6). The poorer plant growth and yield at the clay-loam site were probably due to poorer early growth associated with the cool wet spring, followed by a greater susceptibility to the dryer conditions that followed, due to poorer root development.

6.5. SUMMARY AND CONCLUSIONS

The stimulative effect of Ge on nodulation, nitrogen fixation and growth of soybean plants under field conditions is well documented, however, there has been no previous study investigating the use of *B. japonicum* inoculum induced with MeJA alone or in combination with Ge on soybean nodulation and nitrogen fixation. In this experiment we provide data, from two different field experiments conducted on different soil types with contrasting physical properties and different planting dates, to demonstrate the stimulative effects of MeJA alone or in combination with Ge on nodulation, nitrogen fixation and grain yield by soybean plants. Site had large effects on the ability of plants to produce nodules, fix nitrogen, accumulate biomass and grain yield. Plants growing at the sandy-loam site produced more nodules with more nodule dry weight, leading to enhanced nitrogen fixation.

In general, the results indicate that *B. japonicum* inoculant pre-induced with either Ge, MeJA or both inducers together caused increased nodule number, and nodule dry matter accumulation thereby enhancing nitrogen fixation and grain yield. The results of this study show that the simulative effect of MeJA alone or in combination with genistein was at least generally consistent at both sites, irrespective of their contrasting physical and chemical properties and planting dates. Given that these finding occurred on two sites with different soils and different planting dates the findings appear to be quite robust with regard to environmental conditions.

Table 6.1. Effect of pre-incubation of *Bradyrhizobium japonicum* with genistein (Ge 20µM) and methyl jasmonate (MeJA 50µM) alone or in combination on soybean nodule number and dry weight at two sites with contrasting soil types and different planting dates.

		Sandy-loam site				Clay-loam site			
		Nodule number plant^{-1}		Nodule dry weight (mg)		Nodule number plant^{-1}		Nodule dry weight (mg)	
STRAIN	**INDUCER**	**V3 stage**	**R3 stage**	**V3 stage**	**R3 stage**	**V3 stage**	**R3 stage**	**V3 stage**	**R3 stage**
532C	Ge 20µM	24.1	69.0	123.4	311.3	11.8	17.9	30.0	144.5
	MeJA 50µM	22.7	68.8	122.0	314.8	12.5	17.4	29.0	153.9
	Ge+MeJA	24.1	81.0	127.7	320.3	14.0	18.6	45.0	165.9
	Control (Inoculant Only)	14.7	65.8	97.1	288.8	7.4	12.4	21.7	125.8
USDA3	Ge 20µM	27.2	75.3	113.6	309.3	10.3	17.5	34.5	136.4
	MeJA 50µM	25.4	76.3	114.1	316.0	10.7	18.0	38.8	140.3
	Ge+MeJA	23.4	67.5	120.1	327.3	12.9	22.5	39.2	135.9
	Control (Inoculant Only)	13.8	58.3	85.3	293.5	6.1	12.05	18.2	120.0
Contrasts:									
Inducers versus control		***	**	***	**	***	***	**	**
532C versus USDA3		NS	NS	*	NS	NS	NS	NS	*
532C: Inducers versus control		***	NS	***	*	***	**	*	**
USDA3: Inducers versus control		***	**	***	*	***	***	**	NS

Contrast: NS – Not Significant, *, **, *** - significant at 5, 1 and 0.1 percent level of significance

Table 6.2. Effect of pre-incubation of *Bradyrhizobium japonicum* with genistein (Ge 20µM) and methyl jasmonate (MeJA 50µM) alone or in combination on soybean leaf area and dry matter accumulation at two sites with contrasting soil types and different planting dates.

		Sandy-loam site				Clay-loam site			
		Leaf area plant^{-1} (cm^2)		Dry weight plant^{-1} (g)		Leaf area plant^{-1} (cm^2)		Dry weight plant^{-1} (g)	
STRAIN	**INDUCER**	**V3 stage**	**R3 stage**	**V3 stage**	**R3 stage**	**V3 stage**	**R3 stage**	**V3 stage**	**R3 stage**
532C	Ge 20µM	241	1195	3.20	15.07	235	1001	2.45	13.71
	MeJA 50µM	240	1034	3.34	15.24	214	957	2.28	14.37
	Ge+ MeJA	286	1326	3.54	16.42	222	993	2.32	13.27
	Control (Inoculant only)	199	957	2.54	12.82	207	1010	1.71	11.45
USDA3	Ge 20µM	262	1468	3.24	15.55	245	1018	2.58	13.85
	MeJA 50µM	282	1361	3.55	15.79	229	1090	2.42	13.1
	Ge+ MeJA	255	1250	3.25	16.14	262	1101	2.64	14.48
	Control (Inoculant only)	224	1065	2.65	13.35	207	995	2	11.00
Contrasts:									
All inducers versus control		***	***	***	***	*	NS	***	***
532C versus USDA3		NS	**	NS	NS	NS	NS	NS	NS
532C: Inducers versus control		***	**	***	**	NS	NS	**	***
USDA3: Inducers versus control		**	**	**	**	*	NS	**	***

Contrast: NS – Non significant, *, **, *** - significant at 5, 1 and 0.1 percent level of significance

Table 6.3. Effect of pre-incubation of *Bradyrhizobium japonicum* with genistein (Ge 20µM) and methyl jasmonate (MeJA 50µM) alone or in combination on soybean shoot nitrogen content (Kg ha^{-1}) and total fixed nitrogen (Kg ha^{-1}) at R3 developmental stage at two sites with contrasting soil types and different planting dates.

		Sandy-loam site		Clay-loam site	
STRAIN	**INDUCER**	**Shoot N yield (Kg ha^{-1})**	**Total N fixed (Kg ha^{-1})**	**Shoot N yield (Kg ha^{-1})**	**Total N fixed (Kg ha^{-1})**
532C	Ge 20µM	217.45	124.17	191.72	91.63
	MeJA 50µM	224.00	122.51	206.76	98.86
	Ge+ MeJA	224.05	124.86	190.19	92.73
	Control (Inoculant Only)	198.62	115.51	172.62	82.64
USDA3	Ge 20µM	221.22	120.68	198.99	95.36
	MeJA 50µM	217.08	121.88	183.74	90.16
	Ge+ MeJA	237.81	132.44	207.85	99.34
	Control (Inoculant Only)	205.02	110.28	168.54	80.67
Contrasts:					
Inducers versus control		***	**	***	***
532C versus USDA3		NS	NS	NS	NS
532C: Inducers versus control		**	NS	**	**
USDA3: Inducers versus control		**	**	***	***

Contrast: NS – Not Significant, **, ** *- significant at 1 and 0.1 percent level of significance

Table 6.4. Effect of pre-incubation of *Bradyrhizobium japonicum* with genistein (Ge 20μM) and methyl jasmonate (MeJA 50μM) alone or in combination on soybean seed nitrogen yield (Kg ha^{-1}) and nitrogen fixed (Kg ha^{-1}) at the harvest maturity stage (R8) of soybean plants at two sites with contrasting soil types and different planting dates.

		Sandy-loam site		Clay-loam site	
		Seed N yield	Seed N fixed	Seed N yield	Seed N fixed
STRAIN	INDUCER	(Kg ha^{-1})	(Kg ha^{-1})	(Kg ha^{-1})	(Kg ha^{-1})
532C	Ge 20μM	306.75	181.02	235.75	118.513
	MeJA 50μM	310.01	182.70	239.00	120.178
	Ge+ MeJA	317.75	187.46	241.00	121.138
	Control (Inoculant Only)	273.25	161.08	216.00	108.530
USDA3	Ge 20μM	308.75	182.33	236.50	118.883
	MeJA 50μM	304.25	179.31	239.25	120.358
	Ge+ MeJA	315.75	186.30	246.75	124.110
	Control (Inoculant Only)	284.75	168.17	219.75	110.320
Contrasts:					
Inducers versus control		***	***	***	***
532C versus USDA3		NS	NS	NS	NS
532C: Inducers versus control		***	***	***	***
USDA3: Inducers versus control		***	***	***	***

Contrast: NS – Not Significant, ** *- significant at 0.1 percent level of significance

Table 6.5. Effect of pre-incubation of *Bradyrhizobium japonicum* with genistein (Ge 20µM) and methyl jasmonate (MeJA 50µM) alone or in combination on soybean plant height, yield and yield components on the sandy loam soil

Strain	Inducers	Plant Height (cm)	Per plant		Seeds per Pod	100-seed Weight (g)	Grain Yield (kg ha^{-1})
			Pods	Seeds			
532C	Ge 20µM	77.925	24.9	63.75	2.60	18.78	3823
	MeJA 50µM	80.05	25.0	67.03	2.73	19.38	4047
	Ge+MeJA	78.6	27.9	69.35	2.50	19.28	4009
	Control (Inoculant only)	81.73	20.9	51.83	2.50	18.28	3446
USDA3	Ge 20µM	79.03	24.1	68.2	2.83	18.38	3751
	MeJA 50µM	82.38	23.7	65.2	2.78	19.45	3786
	Ge+MeJA	74.91	26.0	65.1	2.58	19.15	4129
	Control (Inoculant only)	83.18	20.7	51.1	2.48	17.83	3527
Contrasts:							
All inducers versus control		NS	**	***	NS	***	**
532C versus USDA3		NS	NS	NS	NS	NS	NS
532C: Inducers versus control		NS	*	**	NS	**	***
USDA3: Inducers versus control		NS	NS	**	NS	**	**

Contrast: NS – Non significant, *, **, *** - significant at 5, 1 and 0.1 percent level of significance

Table 6.6. Effect of pre-incubation of *Bradyrhizobium japonicum* with genistein (Ge 20µM) and methyl jasmonate (MeJA 50µM) alone or in combination on soybean plant height, yield and yield components on the clay loam soil

Strain	Inducers[a]	Plant Height (cm)	Per plant		Seeds Per Pod	100 seed Weight (g)	Grain Yield (kg ha^{-1})
			Pods	Seeds			
532C	Ge 20µM	52.9	22.15	58.75	2.65	13.0	2397
	MeJA 50µM	53.6	24.33	63.15	2.70	13.8	2371
	Ge+MeJA	53.5	21.95	58.15	2.68	13.2	2396
	Control (Inoculant only)	52.5	20.03	50.70	2.55	11.9	2206
USDA3	Ge 20µM	52.8	23.25	62.00	2.65	12.9	2217
	MeJA 50µM	54.9	22.73	59.25	2.62	13.1	2245
	Ge+MeJA	53.9	23.78	64.53	2.72	13.8	2226
	Control (Inoculant only)	52.7	21.05	53.63	2.58	11.7	1945
Contrasts:							
All inducers versus control		NS	*	**	NS	***	***
532C versus USDA3		NS	NS	NS	NS	NS	**
532C							
Inducers versus control		NS	NS	*	NS	***	*
USDA3							
Inducers versus control		NS	NS	*	NS	***	**

Contrast: NS – Non significant, *, **, *** - significant at 5, and 1 and 0.1 percent level of significance

Figure 6.1 **Daily temperature and precipitation data during the 2002 growing season.**

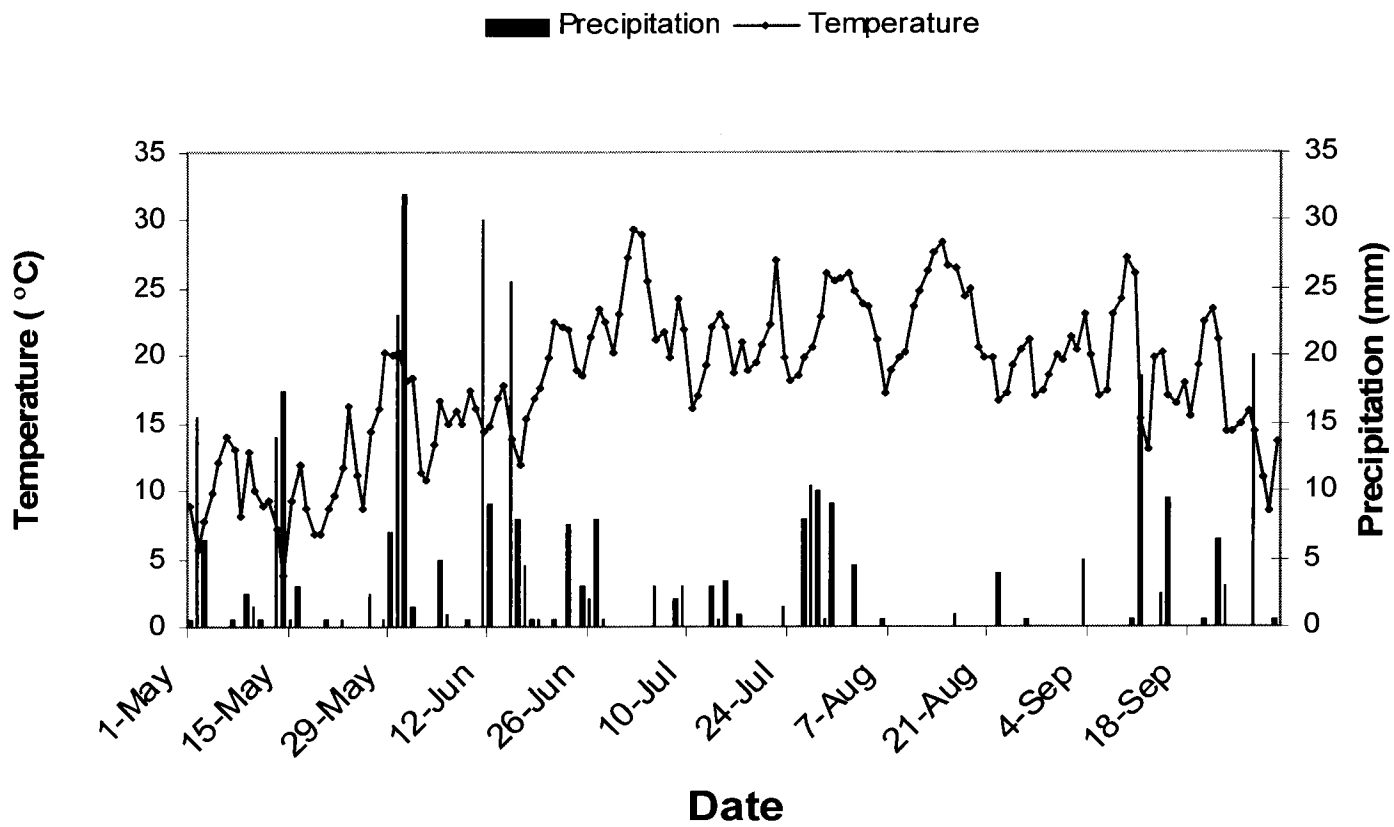
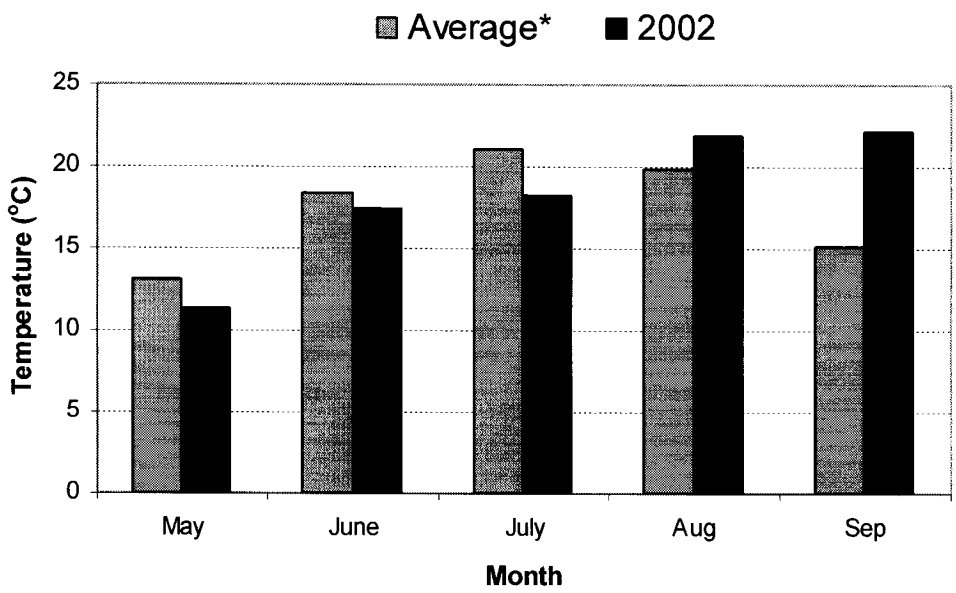
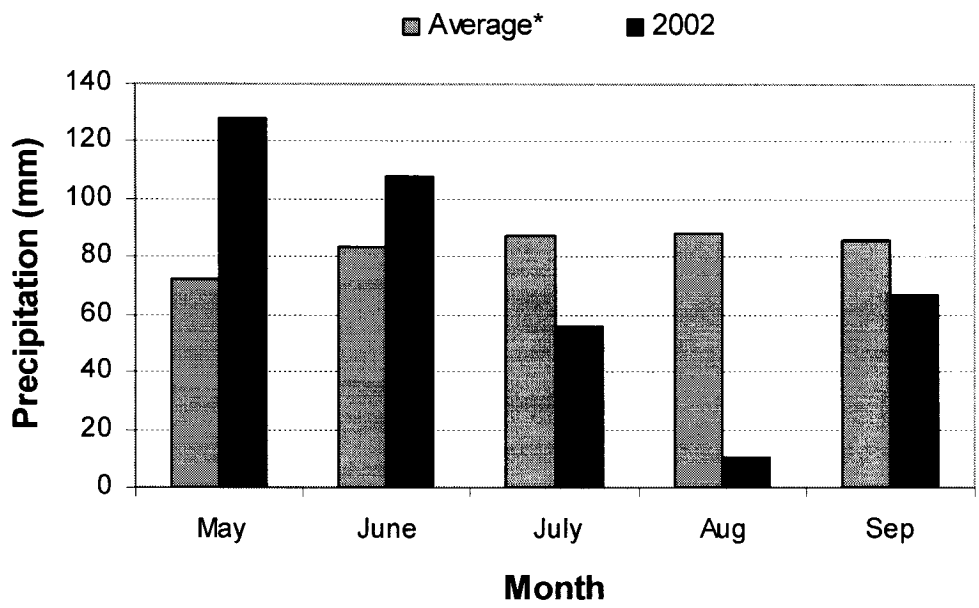

Precipitation
Temperature
Temperature (°C)
Precipitation (mm)
35
30
25
20
15
10
5
0
1-May
15-May
29-May
12-Jun
26-Jun
10-Jul
24-Jul
7-Aug
21-Aug
4-Sep
18-Sep
Date

Figure 6.2 **Monthly average temperature and total monthly precipitation during the 2002 growing season and 57 years average (1945-2002).**





Average* = average of 57 years (1945-2002)

Section 7
GENERAL DISCUSSION

The rhizosphere contains a complex and diverse group of prokaryotes that interact with plant roots (Dobbelaere *et al.,* 2003), in at least some cases, using signal molecules for communication with the plant (Hungria and Stacey, 1997). It now seems likely that this network of signal based communication plays a major role in determining the presence, population and activity of the microbes in the rhizosphere. Billions of microbes can be found in one gram of rhizosphere soil, and is seems probable that many are sending and/or receiving a range of signal molecules to and from the plant roots and neighboring microorganisms. Of this complex and multidimensional intercommunication network, one of the best studied cases of inter-organismal signaling is that of rhizobia-legume signaling network (Broughton *et al.,* 2003). Because of successful signal communication between the two symbionts root hair infection occurs, leading to the biosynthesis of nitrogen fixing nodules.

As an initial step during rhizobia-legume interactions, plant roots exude signals that can trigger the genes essential for the biosynthesis of the rhizobial return signals (Nod factors), deemed necessary for the early steps of root infection. The most widely studied signals that legume roots exude are the flavonoids, a diverse group of compounds, which differ considerably among legume species. The first *nod* gene inducer compound discovered was luteolin (Peters *et al.,* 1986) and subsequent research made it clear that different flavonoids modulate regulation of *nod* genes in different rhizobia.

Recent research findings have demonstrated that some non-flavonoid molecules can also act as *nod* gene inducers in rhizobia. Among the non-flavonoid inducer molecules are the jasmonates. Their potential role in this capacity was only recently reported by Rosas *et al.* (1998), who found that this class of signaling molecules can induce *nod* genes of the fast growing *Rhizobium meliloti.* Although the role of jasmonates is relatively well understood in plant responses to wounding, biotic and abiotic stresses and fungal elicitors, their role in the rhizosphere has been a neglected area of research. Studying the role of jasmonates in rhizosphere biology and biochemistry is thus an extremely intriguing area of research. Since *B. japonicum* is a slow growing rhizobial

species, is physiologically is quite different from fast growing rhizobia such as that worked with by Rosas *et al.* (1998), I conducted several research studies to explore how this class of compounds interacts with *B. japonicum* in a soybean dominated rhizosphere.

The first research question I addressed was "Do jasmonates act as signal molecules for *B. japonicum*?" To address this objective, I used two *B. japonicum* strains (532C and USDA3*)* induced with JA and MeJA at optimum concentrations. In a previous study (Mabood and Smith, unpublished data), using *B.* japonicum strain carrying *nodY::lacZ* fusion, I found that the optimum concentration of JA and MeJA, for the induction of nodulation genes is 50 μM. I included a negative (a blank) and a positive control (genistein at optimal concentration) in this experiment. The results demonstrated that both genistein and jasmonates (JA and MeJA) induced Nod factor production from the tested strains. The Nod factors were isolated and quantified using HPLC and mass spectrometry. I studied the four LCOs most abundantly produced by *B. japonicum* strains 532C and USDA3 following the application of jasmonates. Genistein, JA and MeJA induced the biosynthesis of all four Nod factors; however, JA and MeJA preferentially induced the biosynthesis of some Nod factors that were produced in less abundance in cultures induced only with genistein. Interestingly, there was synergy between the two types of inducer molecules. *B. japonicum* cultures induced with Ge plus JA or MeJA produced more Nod factor than Ge, JA or MeJA alone. The structure of the isolated Nod fators was confirmed using GC/MS.

I found it very interesting that JA and MeJA can act as signaling molecules in *B. japonicum* by inducing the biosynthesis of LCOs. Thus, I explored the biochemistry and molecular biology of jasmonate regulation in plants. Jasmonates are ubiquitously found in plants and biosynthesized from linolenic acid via the octadecanoid pathway. Since jasmonates are fatty acids derived from linolenic acid, I hypothesized that this fatty acid precursor could also induce the expression of *nod* genes and biosynthesis of LCOs. To prove this, I used *nodY::lacZ* fused *B. japonicum*, induced with different concentrations of linolenic and linoleic acids and measured the expression of *nod* genes using the enzyme β-galactosidase. Lipo-chitooligosaccharides were quantified from a wild type strain of *B. japonicum* using HPLC and mass spectrometry. The results of this experiment led me to the discovery that both linolenic and linoleic acids can induce *nod* genes and

cause the biosynthesis of LCOs from *B. japonicum*. These findings demonstrate that fatty acids and their octadecanoid pathway products (JA and MeJA) are a new class of signal molecules in the *Bradyrhizobium*-soybean symbiosis, since it meets the basic criteria of signaling molecules in rhizobia-legume symbioses in that they induce the *nod* genes at low concentrations.

Previous published reports from our laboratory demonstrated that soybean plant growth and development is inhibited by low RZT, and this is particularly so for nodulation and nitrogen fixation (Lynch and Smith, 1993; Zhang and Smith, 1994). Low RZT interferes the early inter-organismal signal exchange between the two symbionts, and delays nodule organogenesis and the onset of nitrogen fixation by soybean plants. It has been reported that low RZT inhibits the biosynthesis and rhizosecretion of genistein, a plant-to-bacteria signal molecule that induces the expression of *B. japonicum nod* genes (Zhang and Smith, 1996a) and the biosynthesis of Nod factors (Zhang *et al,* 2002; McKay, Djordjevic, 1993; Duzan, 2004).

The infection process, during the early stages of nodulation, is also negatively affected by low RZT. However, when genistein is added to bacterial inoculants before they are applied to seedlings (controlled environment conditions) or seeds in furrows (field conditions) this partially alleviates the detrimental effects of low RZT on nodulation and nitrogen fixation by soybean plants (Zhang and Smith, 1996b). Since jasmonates induced Nod factor production from *B. japonicum* and when applied simultaneously with genistein showed synergism; it was reasonable to hypothesize that pre-incubation of *B. japonicum* with jasmonates could enhance nodulation and nitrogen fixation of soybean plants at low RZTs. I conducted a greenhouse experiment to study the effect of *B. japonicum,* induced with JA and MeJA, on soybean nodulation, nitrogen fixation and plant growth. My results demonstrated that MeJA enhanced nodulation and nitrogen fixation at both optimal and sub-optimal RZT in a fashion similar to genistein.

It has been well documented that the soybean crop experiences sub-optimal RZTs during the early stages of plant growth and development in Southern Quebec. This is extremely important since infection and nodulation occur at this stage of plant growth. The results of our greenhouse experiment demonstrated that JA and MeJA could partially alleviate the low RZT induced inhibitory effect on nodulation and nitrogen fixation. As a

follow up to the controlled environment work, I conducted two separate field experiments on two soil types with contrasting physical and chemical properties. These experiments further investigated the effect of MeJA induced *B. japonicum* on soybean nodulation and nitrogen fixation, examining these effects under the much more complex and demanding conditions of the field. I found that *B. japonicum* are more efficient at nodulation, nitrogen fixation and plant growth when pre-incubated with MeJA alone or in combination with genistein. This increase in nodulation and nitrogen fixation produced more grain yield when compared to the control plants, inoculated with non-induced *B. japonicum*. The effect of MeJA with genistein was sometimes synergistic.

Soybean roots produce jasmonates and are exuded into the rhizosphere (Creelman and Mullet, 1995) and I have found that this can lead to induction of *B. japonicum nod* genes resulting in the biosynthesis of Nod factors and enhance nodulation and nitrogen fixation. Thus, this thesis documents the role of jasmonates and their lipid precursors as a new class of signaling molecules in the *Bradyrhizobium*-soybean symbiosis, and demonstrates that they can help to overcome low RZT stress in the same way as flavonoid plant-to-rhizobia signals. In general, the research findings described in this thesis document the discovery of fatty acids and their octadecanoid pathway derivatives, JA and MeJA, as *nod* gene inducers of *B. japonicum*. This thesis also describes jasmonate based formulations and methods to enhance soybean nodulation and nitrogen fixation that have promising commercial applications in agricultural systems.

The agricultural applications are particularly interesting as a flavonoid based technology already exists and has been in the market place for several years. However, the use of flavonoids in commercial production presents two challenges. First, they are very expensive (even when purchased in industrial quantities they are on the order of \$100 g^{-1}. Second, flavonoids often act as phytoallexins in plant responses to pathogenic attacks (Dakora and Phillips, 1996; Dixon *et al.,* 1995) and at the higher concentrations of genistein required to induce *B. japonicum* cells at low RZTs (20 μM) they slow the development of *B. japonicum* cultures, adding cost to commercial production practices. Jasmonates are far less expensive (on the order of \$0.30 per g) and are not harmful to *B. japonicum* cells.

Section 8
CONCLUSIONS

Based upon the research findings of this thesis, a range of general and specific conclusions may be drawn. They have been placed into four general groups addressing specific aspects of *Bradyrhizobium*-soybean signaling, nodulation and plant growth.

1. Jasmonates as signaling molecules in the *Bradyrhizobium*-soybean symbiosis

1.1. JA and MeJA induce Nod factor production by *B. japonicum*.

1.2. JA and MeJA induce Nod factor production by *B. japonicum* to a greater degree than genistein.

1.3. Jasmonates plus genistein together caused greater Nod factor production by *B. japonicum* than either one alone.

1.4. Jasmonates are a new class of signaling molecules in the *Bradyrhizobium*-soybean symbiosis.

2. Linolenic and linoleic acids and *nod* gene induction

2.1. Fatty acid precursors of the octadecanoid pathway (LnA and LcA) induce *nod* genes and cause LCO production by *B. japonicum.* This documents the discovery of a new class of *nod* gene inducer molecules.

2.2. While fatty acids induce *nod* gene activity, they are less effective than genistein and jasmonates.

2.3. Genistein, an isoflavonoid inducer molecule, is a hydrophobic molecule that dissolves poorly in water. Root production of fatty acids as inducer molecules could have, at least, two benefits in the rhizosphere during rhizobium-legume signaling. It could induce *nod* genes and could also serve as a solvent for hydrophobic *nod* gene inducing molecules such as genistein and diadzein.

3. Jasmonates and soybean nodulation and N_2 fixation at low RZT under greenhouse conditions

3.1. Jasmonate pre-induced *B. japonicum* increases plant growth and development, leaf area and dry matter accumulation.

3.2. *B. japonicum* pre-induced with jasmonates increases nodulation and nitrogen fixation leading to increased nitrogen translocation from root nodules to shoots, thereby increasing photosynthesis. Thus, this enhancement of photosynthesis can result in enhanced dry matter accumulation.

4. Jasmonates and soybean nodulation and N_2 fixation under field conditions

4.1. Application of *B. japonicum* pre-induced with MeJA and/or genistein enhances nodulation and nitrogen fixation of soybean plants under field conditions.

4.2. Application of *B. japonicum* pre-induced with MeJA and/or genistein increases dry matter accumulation and grain yield of soybean plants under field conditions.

4.3. The effects of added *nod* gene inducers can be modulated by environmental conditions such as soil type.

4.4. The addition of methyl jasmonate, alone or together with genistein, has potential as a technology for commercial application in the high latitude soybean production areas of the world.

Section 9

ACCEPTANCE AND REJECTION OF HYPOTHESES

Hypothesis 1: Jasmonates cause LCO production by *B. japonicum*

Results related to hypothesis 1: The products of the *nod* gene expression are Nod factors, structurally known as lipo-chitooligosaccharides – LCOs; we induced wild type strains of *B. japonicum* with JA and MeJA and measured LCO production using HPLC and mass spectrometry. Both JA and MeJA induced LCO production by the tested strains (Section 3). Thus, **we accept hypothesis 1.**

Hypothesis 2: Fatty acid precursors of the octadecanoid pathway induce nodulation genes of *B. japonicum*

Results related to hypothesis 2: Jasmonates are produced from linolenic acid and linoleic acids via the octadecanoid pathway in plants. Since Jasmonates induced the expression of *nod* genes of *B. japonicum*, we treated *B. japonicum* strains harboring plasmids carrying *nodY::lacZ* fusions with these fatty acids and then attempted to measure *nod* gene expression activity (β-galactosidase) and to quantify LCO production. We found that both linolenic and linoleic acids induced the transcription of *nod* genes and caused LCO production by *B. japonicum* (Section 4). Thus, **we accept hypothesis 2.**

Hypothesis 3: Jasmonates enhance nodulation, nitrogen fixation and plant growth under sub-optimal RZT conditions

Results related to hypothesis 3: It has previously been shown that induction of *B. japonicum* cells with genistein enhances nodulation, nitrogen fixation and plant dry matter accumulation under low RZT conditions. Since JA and MeJA induced *nod* genes and caused Nod factor production by *B. japonicum,* we conducted a greenhouse experiment to study the ability of JA and MeJA to enhance soybean nodulation, nitrogen fixation and plant growth under greenhouse conditions. We found that both JA and MeJA

enhanced nodulation, nitrogen fixation and plant growth at both optimal and sub-optimal root zone temperature (RZT) conditions (Section 5). Thus, **we accept hypothesis 3.**

Hypothesis 4: Pre-incubation of *B. japonicum* cells with MeJA alone or in combination with genistein enhance nodulation, nitrogen fixation and grain yield of soybean plants grown under field conditions

Results related to hypothesis 4: Since MeJA induced *B. japonicum* enhanced nodulation and nitrogen fixation under greenhouse conditions, we conducted a field experiment to see if MeJA induced *B. japonicum* also enhance soybean nodulation, nitrogen fixation, plant growth and grain yield under the much more complex and demanding conditions present in the field. Our field results demonstrated that MeJA enhanced nodulation, nitrogen fixation, plant growth and grain yield under field conditions, although this was strongly modulated by soil type (Section 6). Thus, **we accept hypothesis 4.**

Section 10
CONTRIBUTIONS TO KNOWLEDGE

The following are considered to be original contributions to knowledge following from the work in this thesis.

1. JA and MeJA cause Nod factor production by *B. japonicum*
2. Addition of optimal concentrations of JA or MeJA, along with genistein, to *B. japonicum* cultures caused synergistic effects on the production of Nod metabolites (LCOs).
3. Jasmonates are stronger inducers of *B. japonicum nod* genes than the isoflavonoids
4. Fatty acid intermediates of the octadecanoid pathway (linolenic and linoleic acids) induce the transcription of *B. japonicum nod* genes
5. Fatty acid intermediates of the octadecanoid pathway (linolenic and linoleic acids) induce LCO production by *B. japonicum*
6. At low RZT, under greenhouse conditions, jasmonate induction of *B. japonicum* cells used as inoculants reduced the low RZT induced inhibitory effects on soybean nodulation and nitrogen fixation.
7. MeJA induced *B. japonicum* can increase nodulation, nitrogen fixation and yield of soybean plants under short season field conditions.
8. Jasmonates and their fatty acid precursors are a new class of signaling molecules in the *Bradyrhizobium*-soybean symbiosis. In fact, this is the first demonstration of *nod* gene induction by these fatty acids in any type of rhizobia.

Section 11
SUGGESTIONS FOR FUTURE RESEARCH

In order to expand the research undertaken in this thesis and to enhance and further our knowledge regarding the role of jasmonates in *Rhizobium*-legume signaling, the following research areas are identified for future work, encompassing research areas from basic to practical agricultural production systems.

1. Dissecting jasmonate mediated regulation of *nod* gene induction in *B. japonicum*

In chapter 3 of this thesis, I showed that jasmonates are able to induce the biosynthesis of Nod factor from *B. japonicum*. This unique role of jasmonates in the positive regulation of the *B. japonicum nod* genes raises several questions. For instance, do these signal molecules induce *nod* genes in *B. japonicum* via the currently recognized three components (NodD1, NodV W - phosphorylated and unphosphorylated form, and nolA – nolA1, nolA2 and nolA3) of induction systems by affecting them differently than flavonoid signals such as genistein? Or, do jasmonates use an alternative set of receptor/*nod* gene regulator proteins? Our findings suggest that jasmonates and genistein activate *nod* genes via different mechanisms because the simultaneous addition of jasmonates with genistein resulted in greater *nod* gene activation (β-galactosidase activity) than when either was added alone, suggesting the activation of, at least, two regulatory mechanisms. The *nod* gene regulatory model described by Loh *et al.* (1997; 1999) indicates that *nod* gene expression is modulated by *nodD* and *nodVW,* while *nolA* constitutes a different signal recognition mechanism. It will be interesting to determine how these different regulatory mechanisms are affected by jasmonate signal molecules. The already known regulatory pathways in *B. japonicum* can be studied as a beginning to answering this question. Studies of strains with deleted *nodD*, *nodV*, *nodW*, *nolA* and/or deletions of combinations of these regulatory genes may help to elucidate the regulatory mechanism of these unique classes of signal molecules in *B. japonicum*. This could further explain the complex regulatory induction mechanism of *nod* genes in *B. japonicum*.

2. **Investigating the role of JA on seed germination, and root growth of legume crops at concentrations that the seeds would receive in an inoculant**

The findings of this thesis have important commercial applications since they provide information on formulation and methods of enhancing soybean nodulation, nitrogen fixation and yield using jasmonate based signal technology. One method of jasmonate based signal technology is direct application to seeds, as a component of liquid inoculants, prior to seeding. Research studies need to be undertaken to determine the effects of jasmonate concentrations that the seeds would receive in the inoculant on soybean seed germination, seedling growth and root development. There is a report that jasmonates can promote root growth and lateral root initiation at sub-micromolar concentrations when applied to tomato roots (Tung *et al.*, 1996).

3. **Are the amounts of jasmonates excreted into the rhizosphere sufficient to cause *nod* gene induction?**

Jasmonates are biosynthesized and excreted into the rhizosphere by legume roots and we have shown that they can induce *nod* genes in *B. japonicum*. However, it is still not clear whether the amounts of jasmonates rhizosecreted into the rhizosphere are sufficient to induce *nod* genes of rhizobia. If they are secreted in sufficient quantity this will raise questions about specificity. It is well established that legumes secrete specific sets of flavonoid compounds and, in general, the correct rhizobial strains respond to these. It will be interesting to see how jasmonates affect different rhizobia. We need to understand the biological implications of this.

4. **Are jasmonates broken down rapidly in the rhizosphere?**

Jasmonates are lipids and little is known about their breakdown by soil microbes. We need to determine whether jasmonates are at least reasonably stable in the complex environment of the rhizosphere or are rapidly broken down there. Research on this matter would add to our understanding of this new class of rhizobia-legume signaling molecules.

5. Will other fatty acids or jasmonates relatives work?

I have shown that jasmonates and their precursor fatty acids (linolenic and linoleic acids) can induce the *nod* genes of *B. japonicum*. Jasmonates are produced from these precursors via the octadecanoid pathway in plants. Future research is needed to address the role of intermediates of this pathway, such as 13-hydroperoxytrienoic acid (HPOT), allene oxide (AO) and 12-Oxophytodienoic acid (OPDA), on the induction of *B. japonicum nod* genes. Since linolenic acid (9,12,15-Octadecatrienoic acid) and linoleic acid (9,12-Octadecadienoic acid) also induced *nod* genes and caused Nod factor production by *B. japonicum*, it would be interesting to study the role of other fatty acids on the induction of *nod* genes and Nod factor production from *B. japonicum*.

6. Study the role of jasmonates on *nod* gene induction leading to Nod factor production from other rhizobia.

In this thesis, I studied the role of jasmonates primarily on the *Bradyrhizobium*-soybean symbiosis. It would be interesting to study the role of jasmonates on the induction of *nod* genes of other rhizobia. Previous research on the induction of *nod* genes has shown that flavonoids show specificity in *nod* gene induction of rhizobia. A flavonoid that induces *nod* genes in one rhizobial species may inhibit *nod* genes in others. However, jasmonates seem not to behave in this manner. They can induce *nod* genes in *Rhizobium meliloti* (a fast growing species) and *Bradyrhizobium japonicum* (a slow growing species). Further work to investigate this phenomenon should be conducted and should highlight the role of jasmonates in rhizobia-legume signaling in general.

7. Testing the role of jasmonates as a tool in accelerating nodulation and nitrogen fixation in other legume-rhizobia systems

Legume crops, based upon their origin, have different temperature responses for nodulation, nitrogen fixation and plant growth. In this thesis I have provided sufficient data to demonstrate that the use jasmonates alone on in combination with genistein can

improve nodulation, nitrogen fixation and plant growth in soybean plants, which require 25-30°C for optimum nodule development and activity, and for plant growth. Once the role of jasmonates in the induction of *nod* genes and Nod factor production is studied in other rhizobia, further studies investigating the role of jasmonates alone on in combination with flavonoid inducer molecules in promoting nodulation, nitrogen fixation, growth and yield in other legume crops at normal and suboptimal RZTs would be of interest as a technology to promote legume crop yield. This study would have direct application to legume agricultural production systems.

Section 12

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