Nod Factor Recognition and Response by Soybean (*Glycine max* [L.] Merr) under Abiotic and Biotic Stress Conditions

Short title

Soybean Response to Exogenous Nod Factor Application

By

Haifa Duzan

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Department of Plant Science

McGill University

Montréal, Québec

August, 2003

Your file Votre référence
ISBN: 0-612-98242-4
Our file Notre référence
ISBN: 0-612-98242-4

Dedication

To the soul of my father, Mohamed Duzan

Abstract

Ph.D. **Haifa Duzan** **Plant Science**

Plants possess highly sensitive perception systems by which they recognize signal compounds originating from microbes. These molecular cues play an important role in both symbiotic and pathogenic relationships. Establishment of the soybean (*Glycine max*)-*Bradyrhizobium* symbiosis is orchestrated by specific signal molecules exchanged between appropriate plant and microbe partners: flavonoids as plant-to-bacteria signals, and Nod factor as bacteria-to-plant signals. How this signaling process interacts with stress conditions (abiotic and biotic) is the subject of this thesis. The abiotic stresses were suboptimal growth temperature, low pH, and salinity. Suboptimal growth temperatures affected the ability of the microsymbiont, *Bradyrhizobim japonicum,* to perceive *nod* gene inducers (genistein) and produce Nod factor. Nod Bj-V ($C_{18:1,}$ MeFuc) production by *B. japonicum* strains 523C and USDA110 was strongly affected by suboptimal growth temperature. Nod factor production declined with temperature, from 28 to 15 °C. Strain USDA110 was more affected by decreased temperature than strain 532C. Decreased Nod factor production at low temperature was due to both decreased bacterial growth and lower production efficiency (Nod factor per cell). When a 1:1 mixture of Nod factor Nod Bj-V ($C_{18:1}$, MeFuc) and Nod Bj-V (Ac, $C_{16:0}$, MeFuc) was applied to soybean roots, root hair deformation increased as Nod factor concentration increased under stressfully low temperature and low pH conditions. High salinity stress strongly reduced the root hair deformation caused by Nod factor, and increasing the concentrations of added Nod factor did not over come this. Exogenous application of Nod Bj-V ($C_{18:1}$, MeFuc), from strain

532C, to soybean root systems under two root zone temperatures (RZTs - 17 and 25 °C) reduced the progression of disease (powdery mildew - *Microsphaera difussa*) development on soybean leaves; this effect increased with Nod factor concentration and was greater at 17 than 25 °C RZT. The first reaction in phenylpropanoid pathway is catalyzed phenylalanine ammonia lyase (PAL). PAL activity was induced by Nod Bj-V ($C_{18:1}$, MeFuc) application to cut stems, and the concentration that caused the most rapid PAL induction and greatest reduction of powdery mildew development following root application (the highest concentration tested: 10^{-6} M).

Résumé

Ph.D. **Haifa Duzan** **Plant Science**

Les plantes possèdent des systèmes de détection sensibles avec lesquels elles peuvent identifier des composés sécrétés par les microbes. Ces signaux jouent un rôle important dans les relations symbiotiques et pathogènes. L'établissement de la symbiose du soja (*Glycine max*)-*Bradyrhizobium* est orchestrée par un échange de signaux moléculaire spécifiques entre plante et leurs partenaires microbiens: les flavonoïdes étant sécrétés par la plante et les facteurs Nod par la bactérie. Les travaux présentés ici se rapportent à comment ces signaux intéragissent à différent stress (abiotiques et biotiques). Des températures suboptimales de croissance, l'acidité et la salinité furent sélectionnées comme stress abiotiques. Les températures suboptimales de croissance affectèrent la capacité du microsymbiont *Bradyrhizobium japonicum* à percevoir les flavonoïdes et à produire des facteurs Nod. La production de Nod Bj-V ($C_{18:1}$, MeFuc) par les souches 532C et USDA110 de *B. japonicum* a été fortement affectée par des températures suboptimales de croissance, diminuant avec la température (28 à 15 °C). La souche USDA110 fut affectée davantage par cette diminution que 532C. La diminution de la production des facteurs Nod à basse températures était conséquence d'une réduction de la croissance bactérienne et de la production des facteurs Nod. Lorsqu'un mélange 1:1 des facteurs Nod Bj-V ($C_{18:1}$, MeFuc) et Nod Bj-V (Ac, $C_{16:0}$, MeFuc) fut appliqué aux racines de soja, la déformation racinaire augmenta avec la concentration des facteurs Nod sous des conditions de stress (bas pH et basse températures). Des conditions de salinité élevées menèrent à une forte inhibition de la déformation racinaires provoquée par les

facteurs Nod; cette inhibition ne fut pas renversée par un ajout de facteurs Nod. L'application des facteurs Nod à deux zones de température aux racines (17 et 25 °C) inhiba la progression du développement du mildiou (*Microsphaera difussa*) sur le soja; cet effet augmenta avec la concentration de facteur Nod, se trouvant à son point culminant à 17 °C plutôt qu'à 25 °C. L'activité de la lyase d'ammoniaque de phénylalanine (PAL) a été induite par le facteur Nod, et la concentration causant l'induction de PAL la plus rapide et la plus grande inhibition du développement du mildiou était identique (10^{-6} M).

Acknowledgements

My deepest appreciation goes to my supervisor Professor D. L. Smith for his constant support and supervision throughout my research. I am very grateful for his understanding and encouragement during this stressful time. I am also very thankful for his valuable advice during thesis writing.

I am very grateful to the members of my advisory committee, Dr. Timothy Paulitz, and Dr. Sam Sparace, both former associate professors of the Plant Science Department, and Dr. Philippe Seguin (assistant professor, Plant Science Department) for their valuable advice and input during advisory committee meetings.

I would like to express my gratitude to Dr. Xiaomin Zhou, Dr. Alfred Souleimanov, and, Dr. Balakrishnan Prithiviraj, for their constant help and suggestions during this research.

I would like to thank Dr. Madani for her suggestions and reviewing the experimental setup for the PAL enzyme assay.

I would like to thank the secretarial staff of the Department of Plant Science and the greenhouse coordinator Mr. Richard Smith.

I am very thankful to all my colleagues in the department, Dr. Wajahat Khan, Dr. Ahsan Habib, Dr. Carlos Costa, Dr. Micheline Ayoub, Mr. Fazli Mabood, Mr. Supanjani Supanjani, Mr. Juan Almaraz, and Mr. Philippe Dufresne for his help with the French translation of the abstract.

My deepest thanks and appreciation goes to my beloved family, especially my mother, my children, Asma and Taha who gave me the motivation to pursue my Ph.D.

and last but not least my husband for his unconditional support and encouragement, and for being there for me whenever I needed him; thank you very much.

With great honor I thank the staff members of the Department of Plant Protection, Faculty of Agriculture, Al-Fatah University, Tripoli, Libya for giving me the opportunity to enrolled in the Ph.D. program at McGill University. Also my thanks go to the Ministry of Education, Tripoli, Libya for the financial support.

Table of Contents

Abstract..... iii

Résumé..... v

Acknowledgements..... vii

Tables of Contents..... ix

List of Tables..... xiv

List of Figures..... xv

List of Abbreviations..... xvii

Contributions of Authors..... xviii

Chapter I

1. General Introduction..... 1

Chapter II

Literature Review..... 4

2.1. The soybean crop..... 4

2.2. The root nodule..... 5

2.3. Recognition..... 6

2.4. Plant physiological responses to Nod factor..... 12

2.5. Abiotic factors limiting symbiosis..... 13

2.5.1. The effect of low temperature on the rhizobia-legume symbiosis..... 14

2.5.2. The effect of low pH on the rhizobia-legume symbiosis..... 15

2.5.3. The effect of salinity on the rhizobia-legume symbiosis 16

2.6. Other environmental factors limiting the symbiosis..... 17

2.7. Biotic factors limiting soybean production..... 19

2.8. Hypotheses..... 23

2.9. Objectives..... 25

Preface to Chapter III..... 27

Chapter III

Nod Bj-V ($C_{18:1}$, MeFuc) Production by *Bradyrhizobium japonicum* (USDA110, 532C) at Suboptimal Growth Temperatures and its Biological Activity..... 28

3.1. Abstract..... 29

3.2. Introduction..... 30

3.3. Materials and Methods..... 34

3.3.1. Bacterial strains..... 34

3.3.2. Bacterial culture and growth conditions..... 35

3.3.3 Population density assay..... 36

3.3.4. Nod factor extraction and purification..... 36

3.3.5. The biological activity of Nod Bj-V ($C_{18:1}$, MeFuc)..... 37

3.4. Results..... 39

3.4.1. The effect of incubation temperature on per culture Nod factor production. 39

3.4.2. Effect temperatures on the biological activity of Nod factor..... 40

3.5. Discussion..... 40

Preface to Chapter IV..... 50

Chapter IV

Recognition of *Bradyrhizobium japonicum* Nod Factor by Soybean [*Glycine max* (L.) Merr.] **Root Hairs under Abiotic Stress Conditions**..... 51

4.1. Abstract..... 52

4.2. Introduction..... 53

4.3. Materials and methods..... 57

4.3.1. Bacterial culture..... 57

4.3.2. Nod factor extraction and purification..... 57

4.3.3. Root hair deformation assay..... 58

4.3.4. Soybean root hair responses to Nod factors treatment under, salinity, low pH, and low incubation temperature stress conditions..... 59

4.3.5. Experimental design and statistical analysis..... 61

4.4. Results..... 62

4.4.1. Soybean root hair responses to Nod factor..... 62

4.4.2. Biological activity of Nod factor at low incubation temperature..... 62

4.4.3. Biological activity of Nod factor under low pH conditions..... 63

4.4.4. Biological activity of Nod factor under salinity stress..... 63

4.5. Discussion..... 64

Preface to Chapter V..... 73

Chapter V

Application of Nod Factor Induces Resistance in Soybean Against Powdery Mildew..... 74

5.1. Abstract..... 75

5.2. Introduction..... 76

5.3. Materials and methods..... 80

5.3.1. Plant materials..... 80

5.3.2. Bacterial culture..... 80

5.3.3. Nod factor extraction and purification..... 81

5.3.4. Effect of temperature and Nod factor concentrations on disease intensity.. 82

5.3.5. Assessment of effect Nod factor on phases of pathogen development..... 84

5.3.6. Enzyme assay (PAL)..... 85

5.3.7. Plant materials..... 85

5.3.8. Nod factor treatment..... 85

5.3.9. Enzyme extraction..... 86

5.3.10. PAL enzyme assay..... 86

5.3.11. Statistical analysis..... 87

5.4. Results..... 87

5.4.1. Effects of Nod factor on disease intensity..... 87

5.4.2. Effect of RZTs on disease development..... 88

5.4.3. Effect of Nod factor on fungal growth at 17 °C RZT..... 88

5.4.4. Effect of Nod factor on fungal growth at 25 °C RZT..... 89

5.4.5. PAL response to Nod factor treatment..... 89

5.5. Discussion..... 90

Chapter VI

General Discussion..... 102

Chapter VII

Acceptance or Rejection of Hypothesis and Contributions to Knowledge..... 111

Chapter VII

Suggestions for Future Research..... 114

References 117

List of Tables

Chapter V:

Table 5.1. Effect of Nod factor treatment on the growth and development of powdery mildew on soybean leaves at 17 °C RZT..... 99

Table 5.2. Effect of Nod factor treatment on the growth and development of powdery mildew on soybean leaves at 25 °C RZT..... 100

List of Figures

Chapter II:

Fig.2.1. The structure of Nod factor.....26

Chapter III:

Fig. 3.1. The effect of incubation temperature on Nod Bj-V ($C_{18:1}$, MeFuc) production.....46

Fig. 3.4. HPLC profile of n-butanol extract from *Bradyrhizobium japonicum*.....47

Fig. 3.5. Types of HAD manifested by Nod factor treatments.....49

Chapter IV:

Fig. 4.1. HAD as a function of Nod factor concentrations and at three incubation temperatures..... 69

Fig. 4.2. HAD as a function of Nod factor concentration and at tested pH levels..... 71

Fig. 4.3. The effect of salt stress on Nod factor bioactivity..... 72

Chapter V:

Fig. 5.1. The effect of Nod factor concentratrions and temperature on disease intensity on soybean leaves 97

Fig. 5.2. PAL specific activity as function of time in soybean leaves treated with a range of Nod factor concentrations.....98

Fig 5.3. Effect of Nod factor on disease incidence on soybean leaves at 17 °C RZT.....101

List of Abbreviations

LCO	Lipo-chitooligosaccharide
RZTs	Low root zone temperatures
CFU	Colony forming unit
PAL	Phenylalanine ammonia lyase
ANOVA	Analysis of variance
SE	Standard error
SAS	Statistical analysis system
HAD	Root hair deformations
BTH	Benzol (1, 2, 3) thiadiazol-7-carbothioic acid S-methylester

Contributions of Authors

This thesis has been written in the form of manuscripts to be submitted to scientific journals. This format has been approved by the Faculty of Graduate Studies as outlined in "Guidelines for Thesis Preparation".

The thesis contains three manuscripts (chapters 3 to 5) prepared by myself. Contributions of co-authors are described in this section. The first author on each of the manuscripts is myself. The final co-author on each of the three manuscripts is my supervisor; Professor D.L. Smith, who provided supervision, technical assistance, and valuable suggestions throughout the work, and corrected the resulting manuscripts, including the thesis. Dr. A. Souleimanov is a co-author on all the three manuscripts. He aided me by providing useful suggestions and discussion related to the experimental setup of the research contained in chapter 3, technical guidance in protein quantification for the PAL assay (chapter 5), helping with HPLC analysis for Nod factor purification, and also proofreading the manuscripts. Dr. X. Zhou is a co-author on the manuscripts contained in chapters 4 and 5; she provided useful suggestions regarding experimental setup for the research described in chapters 4 and 5, and helped with the statistical design and analyses and also proofread the manuscripts.

The bulk of the work was initially proposed and performed by myself. I produced all the bacterial cultures used in the research and conducted all Nod factor extractions and purifications. The technical assistance indicated above was in operating the HPLC. All the laboratory work described in chapter three, such as determinations of Nod factor

concentrations, cell density assays, determinations of root hair deformation and microscopic data collection were all performed by me. In chapter four, the bacterial culturing, Nod factor extraction and purification, and determination of root hair deformation were carried out by me. In chapter five, I designed the experimental setup and performed all the greenhouse experiments, including germinating soybean seeds and seedling transplanting, treatment application and collecting data on disease progression and fungal growth. I carried out protein extractions, protein determinations and determination of PAL activity after some initial discussion with Dr. A. Souleimanov (Chapter 5).

Chapter I

General Introduction

Plants possess highly sensitive perception systems by which signals originating from potentially infecting organisms, both symbiotic and pathogenic, can be recognized (Boller, 1995). During the early stages of rhizobia – legume associations, rhizobia excrete signal molecules, Nod factor, which are perceived with high specificity by the appropriate partners, resulting in formation of a specialized compartment, the nodule, where the microsymbiont resides and fixes nitrogen. Nod factor are lipo-chitooligosaccharides, comprised of a chitin oligomer backbone and a fatty acid side chain at the non-reducing end, and also, possibly, "decorations" in the form of a variety of substitutions on the chitin backbone which contribute to specificity regarding the host plant to be nodulated (Schultze and Kondorosi, 1998).

In addition to roles in the early stages of symbiosis, including root hair deformation, nodule initiation, calcium spiking, and medium alkalinization (Schultze and Kondorosi, 1998), Nod factor have been implicated in other, more general, plant physiological responses. Nod factor enhance early growth of soybean [*Glycine max* (L.) Merr.] seedlings (Souleimanov et al., 2002). In non-legume systems, Nod factor has been reported to act as a substitute for auxin and cytokinin causing enhanced cell division and promoting somatic embryo development in Norway spruce (*Picea abies*); the latter has been attributed to the presence of endogenous Nod-factor like molecules, suggesting the ability of Nod factor to suppress embryogenic cell death (Dyachok et al., 2000; Dyachock

et al., 2002). In addition, mycorrhizal colonization of nodulating and non-nodulating soybean was stimulated by Nod factor treatment (Xie et al., 1995).

Nod factor and chitin oligomers induce similar plant responses, expression of early nodulin gene *enod*40, in soybean roots (Minami et al., 1996). Nod factor and chitin oligomer both induce a cytosolic calcium [Ca^{2+}] increase in transgenic soybean cells; both compounds are potent at nanomolar concentrations with most activity being observed for Nod factor possessing a longer chitin back bone (pentameric), while trimeric and dimeric forms induce markedly lower levels of calcium increase. This suggests a vital role for calcium increase in the signal transduction related to Nod factor, with calcium acting as a second messenger (Müller et al., 2000). Similar results have been reported in chitin-challenged transgenic soybean cells, suggesting that the early rapid increase in Ca^{2+} concentration is part of a mechanism by which plants sense and recognize elicitors, leading to activation of defense responses (Mithöfer et al., 1999).

Nod factor and chitin oligomers also induce the expression of genes potentially involved in defense-related reactions against pathogens (Savoure et al., 1997). Modification of Nod factor substitutions resulted in loss of ability to induce cytosolic Ca^{2+} increases, suggesting the presence of specific plant receptors for Nod factor (Müller et al., 2000). However, in soybean roots and suspension cell cultures a chitin-binding protein was identified in the plasma membrane; purified Nod factor [Nod Bj-V ($C_{18:1}$, MeFuc)] acted as a competitive inhibitor of chitin oligosaccharide binding to this protein, suggesting that soybean perceived both molecules at the same binding site, based on a similar structural features, the chitin backbone (Day et al., 2001).

Induction of plant defense reactions is associated with activation of a number of enzymes of the phenylpropanoid pathway, such as the key enzyme phenylalanine ammonia-lyase (PAL); PAL initiates the first reaction in the pathway (Dixon and Paiva, 1995), leading to the production of secondary metabolites, such as phytoalexins, which are characterized by antimicrobial activity (Morrison and Buxton, 1993). Stimulation of PAL activity in soybean by chitin and chitosan oligomers was observed in soybean, suggesting the induction of this defense-related metabolic pathway in soybean plants (Khan et al., 2003).

Abiotic stress factor, such as salinity, low pH, and low root zone temperature can cause poor legume nodulation in the presence of otherwise compatible symbionts. Early events in the symbiosis, such as signal production and excretion, rhizobial attachment, root hair curling, infection thread formation, and nodule initiation are particularly sensitive to these stresses (Tu 1981; Mckay and Djordjevic, 1993; Zhang and Smith, 1996; Hungria and Stacey, 1997; Hungria and Vargas, 2000). The main purpose of this investigation is to study soybean-*Bradyrhizobium* interactions under abiotic and biotic challenges, in particular, the effects of low root zone temperatures on induction of *B. japonicum nod* genes and the subsequent ability to produce Nod factor. Further, the perception and response to Nod factor by soybean under abiotic stress conditions including: suboptimal temperature, low pH, and salinity was investigated. Finally, the work contained in this thesis describes investigations Nod factor effects on a biotic stress, disease development, when plants are under low and optimal root zone temperature (RZT) conditions and also the development of disease response related PAL activity when soybean is exposed to Nod factor.

Chapter II

Literature Review

2.1. The soybean crop

The ability to establish a nitrogen-fixing symbiosis with rhizobia is restricted to legumes, with the exception of some species in the genus *Parasponia* of the Celtidaceae family (Denarie et al., 1992; Systma et al., 2002). Leguminous plants exhibit very diverse morphology, habitat, and ecology, ranging from arctic annuals to tropical trees (Denarie et al., 1992). Many of these are able to form root nodules and fix atmospheric nitrogen, in association with appropriate rhizobia. The cultivated soybean, *Glycine max* (L.) Merr. belongs to the family Fabaceae (Horner et al., 2003).

Soybean is a plant of tropical to subtropical origin and, as such, requires temperatures in the 25 to 30 °C range for optimal growth and symbiotic N_2 fixation (Jones and Tisdale, 1921). At low soil temperatures, legume nodulation and N_2-fixation activity are strongly and negatively affected (Walsh and Layzell, 1986). Therefore, in short season areas low temperature is considered to be a major growth limiting factor for soybean (Whigham and Minor, 1978). Soybean is the world's most widely-produced nitrogen (N_2) fixing crop. In symbiotic association with *Bradyrhizobium japonicum*, soybean can fix 100 to 200 kg ha^{-1} yr^{-1} of atmospheric N (Smith and Hume, 1987).

Soybean is a globally important crop, grown to at least some extent in most parts of the world as a primary source of vegetable oil and protein (Sinclair and Backman, 1989). Annual global production of soybean is on the order of 154 x 10^6 t, of which Canada accounts for approximately 2.8 x 10^6 t (Wrather et al., 2001). Canadian soybean production has been increasing steadily for several decades, and since 1994 there has been sharp increase in Quebec; soybean is sometimes the top field crop in Ontario (McClary, 1997). Total Canadian area planted with soybean increased from 836 000 ha in 1994 to 966 000 ha in 1998 (Wrather et al., 2001).

2.2. The root nodule

Rhizobia are gram negative bacteria capable of reducing atmospheric dinitrogen to ammonia in association mainly with roots of many leguminous plants, forming a highly specialized structure, the nodule, in which biological nitrogen fixation occurs. Nodulation is a multistep infection process that involves specific plant and bacterial gene expression. Before the attachment step, and during the preinfection stages, symbionts must be exposed to each other for a period of time. Bacteria multiply and colonize the root surface and induce root hair tip curling forming pocket-like deformations where they are trapped, providing appropriate attachment for the later steps of infection, penetration into the root hair, forming the infection thread, which is initiated by cell wall invagination and develops toward the base of the infected root hair cells. This is followed by cortical cell division and subsequent nodule meristem development in the cortex. Rhizobia multiply and remain contained within the infection thread which will eventually reach the nodule primordium, where bacteria released into cells of the nodule meristem in the form of

bacteroids, the differentiated form of rhizobia which fixes nitrogen (Schultze and Kondorosi, 1998; Geurts and Bisseling, 2002). The most frequently nodulated region of the root is located above the zone of rapid root elongation and below the smallest emergent root hairs present at the time of inoculation (Bhuvaneswari et al., 1980).

2.3. Recognition

The establishment of compatible symbiotic N_2-fixing associations between rhizobia and legumes is a multi-step process, the success of which is partly determined by preinfection events in the rhizosphere. These events involve recognition between two appropriate partners. The process starts with coordinated mutual exchange of molecular signals between the bacterium and host plant. The first step in this exchange is production and excretion of chemical attractants, especially (iso)flavonoids, from the plant roots, (Schultze and Kondorosi, 1998). These signal compounds are often exuded by the portion of the root with emerging root hairs, a region that is highly susceptible to infection by rhizobia (Verma, 1992). These compounds activate the expression of *nod* genes in rhizobia, stimulating production of bacterial Nod factor (Geurts and Bisseling, 2002). Flavonoid molecules that act as early nodulation signals between legumes and their rhizobial symbiotic partners vary in both kind and relative amount from one type of host plant to another (Kosslak et al., 1987; Appelbaum, 1990).

In the soybean – *B. japonicum* symbiosis the isoflavones genistein and daidzein are recognized as the primary signal molecules between soybean and *B. japonicum nod*

genes (Kosslak et al., 1987; Ip et al., 2001). A variety of other flavonoid compounds can also act as inducers of *nod genes*. Both quercetin and kaempferol, are known inducers of soybean microsymbiont *nod* genes, having 10 and 29%, respectively, of the reported genistein activity (Kosslak et al., 1987). Application of genistein has been shown to increase soybean nodulation and N_2 fixation at suboptimal root zone temperatures (RZTs). The duration of the root hair curling stage is shortened by inoculation with genistein-preincubated *B. japonicum*, especially under low temperature conditions (Zhang and Smith, 1994).

Cho and Harper (1991) reported a strong positive relationship between root isoflavone concentrations and soybean nodule numbers. For instance, a hypernodulating soybean mutant, derived from the cultivar Williams, had higher root concentrations of isoflavone compounds (genistein, daidzein, and coumestrol) than did Williams at 12 days after inoculation.

In response to flavonoids exuded by the host roots, bacterial *nod* gene transcription is stimulated; *nod* genes specify the synthesis of novel signal molecules termed Nod factor (Geurts and Bisseling, 2002). Nodulation genes are usually classified into categories, which include the following: I. the common nodulation genes, *nodA*, *nodB*, and *nodC*,which exhibit homology among *Rhizobium* and *Bradyrhizobium* species and are host inducible and essential for nodulation. They are involved in the biosynthesis of substituted lipo-chitooligosaccharides [LCOs, i.e. Nod factor] (Perret et al., 2000; Wais et al., 2002). Nod factor biosybthesis is intiated by elongation of the non-reducing end, catalyzed by the NodC protein UDP-N-acetyl glucosaminyltransferase, *nodC* also

encodes for ß-glucososyl transferases catalyzing the incorporation of N-acetyl glucosamine into cell wall polysaccharides (Hirsh et al., 2000; Perret et al., 2000). Subsequently, the NodB protein, deacetylase, removes the acetyl group from the non-reducing terminus sugar (Stokkermans et al., 1995), and finally, the NodA protein, acyltransferase catalyzes the addition of a fatty acyl chain by linking the acyl chain to the acetyl-free C-2 carbon of the non-reducing end of the oligosaccharide (Perret et al., 2000). II. *nodD*, an initial regulatory gene that positively regulates the transcription of structural *nod genes* (Stougard 2000). Rhizobia can posses more than one regulatory NodD protein, such as, NodD1 and NodD2, the two component regulatory genes (e.g. *nolA* and *nodVW* in *B. japonicum*) have been identified in *B. japonicum* . In the absence of flavones (inducers), NolA is induced and negatively regulates the nodulation genes by activating the transcription of *nodD2*; the resulting protein product represses the transcription of *nod* genes (Garcia et al., 1996, Loh and Stacey, 2001). III. Genes that determine host range specificity (*hsn*, host-specific nodulation). IV. Genotype specific nodulation (GSN) genes, which appear to specify the ability to nodulate selected genotypes within a legume species. The nodulation genes are localized on a megaplasmid (pSym) in fast growing rhizobia, whereas in *Bradyrhizobium* and *Azorhizobium* they are located on the chromosome (Hungria and Stacey, 1997).

Over 60 Nod factor structures, produced by 17 different rhizobial strains, have been identified (Price, 1999). Each rhizobial species has a characteristic set of nodulation genes that specify the length of the chitooligosaccharide backbone, the type of substitutions along the chitin backbone and the nature (length and degree of unsaturation) of the lipid side chain (Denarie et al., 1996). All these molecules have a similar basic

structure, composed of a chitooligosaccharide (a linear chain of ß-1,4-linked *N*-acetylglucosamines) linked to an acyl chain (Mergaert et al., 1997). In addition to the acyl substitution, several other modifications of the Nod factor molecules have been described for both reducing and nonreducing end residues. For example, carbamoylation and N-methylation of the nonreducing terminal glucosamine is found on Nod factor from *Bradyrhizobium elkanii*, *Rhizobium sp.* NGR234, and *Azorhizobium caulinodans* (Price et al., 1992; Carlson et al., 1993; Mergaert et al., 1993). Additionally, acetylation at *O-6* of this glucosamine residue is reported for the Nod factor from *B. japonicum* and *B. elkanii* (Carlson et al., 1993). A number of modifications of the reducing end residue at *O-6* were identified, such as, sulfation (Truchet et al., 1991), acetylation (Firmin et al., 1993), and fucosylation (Sanjuan et al., 1992; Carlson et al., 1993).

Analysis of the structure-function relationships of various Nod factor molecules has shown that specificity is determined by the various residues present on the core lipo-chitin backbone. For example, in the case of *B. japonicum*, specificity is determined, in part, by the presence of a *2-O*-methylfucose residue attached to the terminal, reducing residue (Sanjuan et al., 1992). When *2-O*-methylfucose is removed from *B. japonicum* Nod factor, complete loss of bioactivity results (Stokkermans et al., 1995). However, Nod factor with a broad variety of structures are active on bean roots. The common Nod factor produced by bean rhizobia, a pentameric Nod factor with an *N*-methyl substituent, is also produced by rhizobia that do not nodulate bean (Hungria and Stacey, 1997). *Bradyrhizobium japonicum* USDA110 and 532C produce one major lipo-chitooligosaccharide (Sanjuan et al., 1992; Souleimanov et al., 2002) and a number of less abundant ones; however, *B. japonicum* strain USDA135 produces several abundant lipo-

oligosaccharides (Spaink et al., 1992). At least two distinct Nod factors [Nod Bj-V ($C_{18:1}$, MeFuc) and Nod Bj-V ($C_{16:0}$, MeFuc)] were found to be produced by the microsymbionts of soybean, *B. japonicum* strains USDA110, and USDA135, and *B. elkanii* strain USDA61. At least two Nod factor are required for induction of early nodulin gene in soybean roots (Minami et al., 1996).

The nomenclature of Nod factors can most easily be explained with an example. In the case of Nod Bj-V ($C_{18:1}$, MeFuc) Bj refers to N_2 fixing microorganism *Bradyrhizobium japonicum*, and V to the length of the chitin backbone, while $C_{18:1}$ refers to the length of fatty acyl moiety with one unsaturation and MeFuc is stand for the type of substitution, methyl fucose, on the chitin backbone (Souleimanov et al., 2002).

In addition to Nod factor, other types of rhizobial signal molecules are involved and required for successful nodulation, for instance, the *nodO* gene of *Rhizobium leguminosarum* bv *viciae* is essential for nodulation and encodes a Ca^{2+}- binding protein required for Ca^{2+} transport via and ion channel, allowing *nod* factor transport across membranes (Stougard et al., 2000). Other types of extracellular proteins, known as nodulation outer proteins (Nops), are excreted by rhizobia upon flavonoid induction. Impaired nodules was formed on *Crotalaria juncea* roots when inoculated with *Rhizobium* sp. NGR234 mutated in *nopX* and *nopL*,genes (Marie et al., 2003), similar results were reported for *Sinorhizobium fredii* USDA257 symbiont of soybean (Krishnan et al., 2003).

The rhizobial structural polysaccharides also appear to be essential signal molecules during the early infection process. Non functional nodule formation was observed when *Vicia sativa* sp. *nigra* was inoculated with exopolysaccharide-deficient mutants (Exo mutants) of *Rhizobium leguminosarum* bv. *viciae* (Vanworkum, et al., 1995), moreover, decreased nodulation competitiveness was also reported in exopolysaccharides-mutated rhizobia (Krishnan and Pueppke, 1998). Enhanced strain competitiveness for nodulation was observed due to the production of trifolitoxin, an antirhizobial peptide produced by some strains of *R. leguminosarum* (Triplett et al., 1994).

Likewise, a number of molecular signals other than (iso)flavonoids are produced by symbiotic legumes and play major roles in nodulation, such as, betains, alkaloids, sugar acids, and amino acids. Rhizobia chemotactic responses toward legume roots are mediated by the amino acids glutamate and aspartate (Ndakidemi and Dakora, 2003). Betaines are a second class signal molecule released by *Medicago sativa* L. root system. They cause the induction of nodulation *genes* in *Rhizobium meliloti*, while, in the *M. littoralis* symbiosis only betains act as *nod gene* inducers (Phillips et al., 1994; 1995). Moreover, rhizobia *nod genes* were induced by alkaloids (trigonelline and stachydrine) exuded by alfalfa roots, while, in lupin sugar acids (erythronic and tetronic acids) acted as a signals for *nod gene* induction (Ndakidemi and Dakora, 2003).

Uridine, ethylene and mimosine are regulators of nodulation process. Cortical cell division is activated by diffusion of uridine from the stele into the cortex in the protoxylem zone of the roots, while, ethylene synthesized in the pericycle and diffuses

into the cortex and blocks cell division (Lazarowitz and Bisseling, 1997). Mimosine, is a non-protein amino acid. It is toxic and is produced by the tree legume *Leuceaena*, allowing higher competitiveness to mimosine degrading rhizobia (Soedarjo and Borthakur, 1998).

2.4. Plant physiological responses to Nod factor

Purified or chemically synthesized Nod factor can elicit various effects on legume plants. Exogenous applications of pure Nod factor have been shown to induce several physiological responses in the plant that are also induced by rhizobial infection. These responses include root-hair deformation, formation of pre-infection threads, cortical cell divisions, alkalinization, and calcium spiking (van Brussel et al., 1992; Schultze and Kondorosi, 1998; Lhuissier et al., 2001). Moreover, in some legume systems the formation of mature structures resembling authentic, although empty (lacking bacteroids), nodules was also observed on Nod factor treated roots. At 10^{-7} M, Nod factor induced pseudo nodule formation on roots of the legume *Macroptilium atropurpureum* (Relić et al., 1993). Chemically synthesized and naturally purified Nod factor from *B. elkanii* strain USDA61 were shown to induce the formation of complete nodule structures on *G. soja*. The most active lipo-chitooligosaccharides induced complete nodular structures at 1 ng of lipo-chitooligosaccharide per spot inoculation (Stokkermans and Peters, 1994; Stokkermans et al., 1995). The formation of full-size nodular structures, including fully formed vascular systems, after induction with Nod factor, has also been shown for *Medicago* plants. For *Medicago*, this exceptional ability might be related to the ability of wild genotypes to form root nodules spontaneously in the absence of added Nod factor

(Truchet et al., 1989). However, *G. soja* is not known to form nodules spontaneously and yet it also has the ability to form nodule structures when its roots are exposed to Nod factor. Thus, it was concluded that the Nod factor alone are sufficient for development of complete nodule structures, at least on some legumes (Stokkermans and Peters, 1994; Stokkermans et al., 1995). Even so, failure of Nod factor to induce complete nodules is reported (Sanjuan et al., 1992), and this may be due to application of insufficient Nod factor (Stokkermans et al., 1995) or plant genotype differences.

Enhanced mycorhizal colonization of non-nodulating and nodulating soybean genotypes, as well as *Lablab purpureus,* has been observed following Nod factor application (Xie et al., 1995; 1997). Moreover, addition of Nod factor to non-host tomato suspension-cultured cell induced alkalinization, a reaction commonly elicited by chitin application (Staehelin et al., 1994). Further more, induced expression of genes encoding the enzymes of the isoflavonoid biosynthetic pathway in *Medicago* roots, including chalcone synthase, chalcone reductase, isoflavone reductase and a pathogen related protein was also reported with Nod factor application (Savoure et al., 1997). In a comparable study, both, chitin oligosaccharides and the main nod factor produced by *B. japonicum* [Nod Bj-V ($C_{18:1}$, MeFuc)] elicited similar phytoactive defense responses in soybean cell cultures (Day et al., 2001).

2.5. Abiotic factors limiting symbiosis

Abiotic stress factors, such as salinity, low pH, and low soil temperature can cause poor nodulation in the presence of otherwise compatible symbionts. Early events in the

symbiosis, such as signal exchange and excretion, attachment, root hair curling, infection thread formation, and nodule initiation, are particularly sensitive to these stresses (Tu, 1981; Mckay and Djordjevic, 1993; Zhang and Smith, 1996; Hungria and Stacey, 1997; Hungria and Vargas, 2000; Duzan et al., chapters three and four).

2.5.1. The effect of low temperature on the rhizobia-legume symbiosis

Low temperature is a major limiting factor for soybean growth and nodulation in areas with relatively short growing seasons (Whigham and Minor, 1978). Early studies showed that root temperatures in the 25 to 30 °C range are optimal for soybean N_2 fixation (Jones and Tisdale, 1921). Low seasonal temperatures (usually May to September) restrict the growth of N_2 fixing soybean in eastern Canada (Lynch and Smith, 1993), affecting all stages of symbiosis establishment including, signal exchange, root hair curling, infection thread formation and penetration, and nodule development and function, with the infection processes being the most sensitive steps (Walsh and Layzell, 1986; Zhang and Smith, 1994). Low root zone temperatures (RZTs) also reduce the N_2 fixation activity of the nitrogenase enzyme complex (Layzell et al., 1984). When the RZT was between 25 and 17° C, the time between inoculation and the onset of N_2 fixation was delayed by 2 days for each degree decrease in temperature, whereas between 17 and 15 °C each degree delayed the onset of N_2 fixation by about 1 week (Zhang et al., 1995). Similar results have been observed in field bean, presumably due to a prolonged pre-infection period (24 days at 10 °C, 8 days at 18 °C) as well as slower differentiation of nodules at the lower temperature (Fyson and Sprent, 1982). Under optimal conditions only 24 h is required for the initiation of infection threads (Fyson and Sprent, 1982).

Suboptimal soil temperature inhibits the release of plant-to-bacteria signal molecules by soybean roots and their perception by rhizobia, resulting in less *nod* gene expression (*nodY-lacZ* fusion of *B. japonicum* USDA110), and, presumably, less Nod factor production as temperature declined from 17 to 15 °C (Zhang and Smith, 1996; Pan and Smith, 1998; Duzan et al., chapter three). Similar results were reported for *R. leguminosarum* bv. trifolii Nod factor production (McKay and Djordjevic, 1993). Low temperature may also limit the rate of fixed nitrogen export, in the form of ureide, from nodules, resulting in accumulation of nitrogen inside the nodule, causing feedback inhibition of N_2 fixation (Sprent, 1979).

2.5.2. The effect of low pH on the rhizobia-legume symbiosis

Nodulation under low pH conditions has been attributed to sensitivity of early events in the symbiosis (Mckay and Djordjevic, 1993). Both, the ability of the host plant to excrete signal molecules and their perception by rhizobia were adversely affected by low pH conditions. The most low pH sensitive stage in the *Rhizobium meliloti* / *Medicago sativa* L association is the curling process (Munns, 1968).

Reductions in the amounts of excreted *nod* gene inducers by subterranean clover (*Trifolium subterraneum* L.) roots was inhibited at a pH of less than 5, consequently, limited induction of *nod* genes was detected in three tested strains of *Rhizobium leguminosarum* biovar *trifolii*. Further restriction was observed at pH 4.8 (Richardson et al., 1988a), and a lower concentration of *nod* gene encoded metabolites was detected,

presumably due to inhibition of Nod factor excretion rather than production; even so, there were differences among tested strains (Mckay and Djordjevic, 1993).

At pH 4.0, *Bradyrhizobium japonicum* A1017 exhibited a greater sensitivity to low pH-stress than *Rhizobium fredii* P220. This was attributed to the ability of the fast growing *R. fredii* to maintain higher contents of homospermidine as well as cellular Mg^{2+} levels (Fujihara and Yoneyama, 1993). Nodule initiation, number, and dry weight for soybean are negatively affected by low pH, with the lowest pH tested, pH 4.5, causing the most negative effects (Alva et al., 1987). Similar results were reported for bean; adding *nod*-gene inducers (genistein) potentially overcomes the stress imposed by low pH growth conditions (Hungria and Stacey, 1997).

Low pH may also be associated with low concentrations of calcium and molybdenum, and toxic levels of aluminum, and manganese (Sprent, 1979). In the presence of calcium, induction of *nod A* in *Rhizobium leguminosarum* bv. *trifolii* was less affected by low pH (Richardson et al., 1988b). An indirect effect of lower pH is decreased phosphate availability; lower Nod factor excretion levels were observed under phosphate limited growth conditions (McKay and Djordjevic, 1993).

2.5.3. The effect of salinity on the rhizobia-legume symbiosis

Salinity stress is a major environmental constraint on rhizobia-legume associations, affecting their capacity for nodulation and N_2 fixation. At high solute concentrations water is withdrawn osmotically from nodules (Sprent, 1979). The growth and

development of root and nodule dry weights of Faba bean were decreased by 100 mM NaCl (Cordovilla et al., 1999). A 50% reduction in soybean nodule number and weight was associated with extreme sensitivity of nodule initiation to salt treatment, and this was attributed to limited root growth and decreases in the proportion of infected root hairs (Singleton and Bohlool, 1984; Saadallah et al., 2001). Inhibition of root hair curling, shrinkage of root hairs, and reductions in nodule numbers were observed for NaCl treatments. Nodulation was completely eliminated at 1.2% (205.6 mM) NaCl (Tu 1981; Ikeda, 1994).

The impact of salinity on the growth and survival of free living rhizobial cells has also been investigated. Generally, rhizobia are less affected by salt stress than their plant hosts (reviewed by Garg and Gupta, 2000). *Rhizobium* starins are more salt-tolerant than strains of *Bradyrhizobium* and differential responses of *Bradyrhizobium sp.* strains were reported to increasing concentrations of NaCl (50-400 mM) (Elsheikh, 1998).

2.6. Other environmental factors limiting the symbiosis

Water supply has a major effect on nodulation and nitrogen fixation. Infection is restricted in dry soils owing the absence of normal root hairs. Instead, short, stubby root hairs develop, which are inadequate for infection by *Rhizobium* (Lie, 1981).

Decreased soil water potential reduces the number of infection threads, root and root hair growth (Sprent, 1979). This effect is reversible when the plants are rewatered; immature hairs resume normal growth and can become infected. Reduced water supply

following successful infection may also retard nodule development, accelerate senescence, and generally lead to lower nitrogen fixation rates (Sprent, 1979), with accompanying reductions in the nodule respiration, transport of fixed nitrogen out of the nodules, and photosynthate supply from a stressed shoot system (Huang et al., 1975).

Nodulation increases with soil water content until waterlogging occurs. Excess water is particularly detrimental to nitrogen fixation as even a thin layer of water on the nodule surface markedly reduces fixation (Lie, 1981). Low availability of oxygen reduces root growth and function, root-hair deformation, nodule development and nodule size; moreover, ethylene is produced in anaerobic soil, which, at very low concentrations, restricts nodulation (Lie, 1981). Wet and oxygen limited soil conditions also provide an optimal environment for the occurrence of root rot organisms such as *Phytophthora megasperma var. sojae* (Hicks, 1978).

In the field, a temporary layer of water around the nodule occurs after any significant rainfall or irritgation event. This can be an important regulator of nitrogen fixation. Under prolonged exposure to water logging, plants may develop special structural changes on the nodule surface, such as, ruptures, protuberance and lenticles (Sprent, 1979).

The supply of essential nutrients in the soil affects both root and nodule development. The most notable effect on nodule development and function is brought about by the presence of mineral nitrogen. Increasing soil mineral nitrogen reduces nodule number, size, and metabolic activity. Nodule number, fresh weight, and size, were

reduced by 33, 50, and 25%, respectively, and less nitrogen was fixed when nitrogen fertilizer was applied (Hicks, 1978). It is clear that mineral nitrogen inhibits the development of the legume-rhizobia symbiosis at many steps, and it has been demonstrated that the production of plant-to-bacteria signals is inhibited (Zhang et al., 2000).

Combined nitrogen [generally nitrate (NO_3), ammonium (NH_4), and urea] has been demonstrated to inhibit all components of symbiotic N_2 fixation. Symbiotic N_2 fixation is affected at all stages, from the initial bidirectional signal exchange between symbionts through to nodule senescence. This has been attributed to a shortage of carbohydrates and/or reducing power necessary for the competing metabolic processes of NO_3^- assimilation and symbiotic N_2 fixation (Waterer and Vessey, 1993). Under field conditions, nitrogen application markedly decreased the concentrations of isoflavonoids, signals excreted by the macrosymbiont, resulting in decreased nodulation (Eardly et al., 1984).

2.7. Biotic factors limiting soybean production

Soybean production worldwide is limited by a number of diseases caused by a range of pathogens, such as, nematodes and fungi. The parasitic cyst nematode (*Heterodera glycines* Ichinohe) is one of the most destructive soybean pathogens in North America. It attack the root system causing considerable yield losses. Serious diseases are also caused by a variety of fungal pathogens, sometimes leading to major yield reductions, for example *Septoria glycines* (Hemmi) is the causal agent of brown spot, *Sclerotinia sclerotiorum* (Lib.) de Bary is the causal agent of sclerotinia stem rot, and *Microsphaera*

difussa Cke. & Pk. is the causal agent of powdery mildew (Wrather et al., 2001). Powdery mildew can disrupt leaf structure, leading to declines in photosynthetic activity as great as 50%, and yield losses of up to 35% in naturally infected fields of susceptible soybean cultivars (Dunleavy, 1979; Mignucci and Boyer, 1979; Phillips, 1984). In Brazil, losses caused by soybean powdery mildew were estimated at 30 to 40% (Gonçalves and Di Mauro, 2002). Recently, serious outbreaks of soybean powdery mildew were reported in eastern Asia (Takamatsu et al., 2002). The earliest reported occurrence of soybean powdery mildew is from the U.S. (Lehman, 1931).

Similar to the symptoms of other mildews, soybean powdery mildew causes irregular dust-like white patches to form on soybean leaf surfaces. The fungus possesses the ability to directly penetrate the cuticle and epidermal cell wall and form a specialized structure, the haustoria, the structure by which the fungus obtains nutrients from its host. The anamorphic stage is characterized by substraight to flexuous hyphal growth and multi to moderately lobed appressoria. Conidiophores produce conidia which are oval, ellipsoidal or cylindrical in shape. Two fungal species were identified as casual agents in soybean powdery mildew, *Erysiphe glycines* in eastern Asia, and *E. difussa* (= *Microsphaera difussa*) in North America, both species are known to produce perfect stage ascomata possessing two differential types of appendages (Takamatsu et al., 2002).

In general, the control of powdery mildew has relied on extensive use of fungicide, however, some of these are environmental concerns, and their continued use inevitably leads to development of resistant strains of the pathogen. Several studies have been conducted to find safe, alternative, environmentally-friendly methods of disease

management, among them, biological control and induced resistance (Singh and Prithiviraj, 1997). When the biocontrol agent *Trichoderma harzianum* (T39) was applied to the cucumber root zone, induced resistance was observed against powdery mildew disease development (Elad, 2000). Similarly, induced resistance against cucumber powdery mildew was reported using Milsana (extract from knotweed) and this was associated with activation of genes encoding biosynthetic enzymes of the phenylpropanoid pathway (Fofana et al., 2002). Recently, induction of systemic protection against barley powdery mildew was confirmed by application of methyl jasmonate (Walters et al., 2002). Systemic acquired resistance was induced against powdery mildew when the synthetic BTH was applied (Heitefuss, 2001).

A key enzyme in the phenylpropanoid pathway is phenylalanine ammonia-lyase (PAL), which initiates the first reaction in the pathway, resulting in the formation of cinnamic acid (Dixon et al., 1995). Cinnamic acid is metabolized to supply intermediates for the synthesis of secondary metabolites such as phytoalexins, which are characterized by antimicrobial activities (Morrison and Buxton, 1993). PAL activity can be induced by abiotic factors and during plant-pathogen interactions (Dixon and Paiva, 1995), however, PAL can be induced during early stages of symbiosis establishment. Infection of soybean roots with *B. japonicum* induces PAL genes (Estabrook et al., 1991). Additionally, Nod factor induced the expression of genes involved in plant defense induction in *Medicago* cell cultures (Savoure et al., 1997). PAL activity can also be stimulated by a number of elicitors, including chitin and chitosan. Recently, the stimulation of PAL activity in soybean was observed in soybean following treatment with chitin and chitosan oligomers,

suggesting the induction of defense-related metabolic pathways in soybean plants (Khan et al., 2003).

2.8. Hypotheses

Successful legume N_2-fixing symbioses require tripartite interactions between the nitrogen fixing microorganism, such as, *Bradyrhizobium japonicum*, and a higher plant, such as, soybean, under favorable environmental conditions. The success of the symbiosis is highly dependant on mutual exchange of specific signal molecules between the two appropriate partners. These early stages, in particular, can be disrupted by a number of abiotic factors, such as, low root zone temperature, low pH, salinity and biotic factors, such as diseases caused by diverse types of plant pathogens.

Symbiosis, in the broad sense, means beneficial and parasitic relationships, in this case between plants and a wide range of microorganisms. The interaction between plant and microsymbiont can be beneficial such as the symbiotic N_2 fixation relationship of legumes, or parasitic. Both types of associations involve induction of similar genes and metabolic pathways (Baron and Zambryski, 1995). In rhizobia-legume associations, Nod factor induce genes encoding enzymes involved in plant defense reactions, which suggests the possible use of Nod factor for induction of plant disease resistance. Thus the hypotheses underlying the research contained in this thesis are:

1. Low RZTs inhibit bacteria-to-plant signaling at the beginning of the establishment of the soybean nitrogen fixing symbiosis, particularly, the ability of the bacteria to produce Nod factor and the ability of the soybean root system to respond to them.

2. Low RZTs, low pH, and salinity affect Nod factor perception by soybean root hairs, affecting their biological activity.

3. Addition of Nod factor to the soybean-bradyrhizobia system potentially induces plant defense responses against soybean powdery mildew.

4. Both, pathogenic infection (powdery mildew on soybean leaves) and controlled infection (symbiosis, root infection by rhizobia) are similarly affected by low RZTs.

2.9. Objectives

1. To test the effects of suboptimal growth temperature on production of the Nod factor [Nod Bj-V ($C_{18:1}$, MeFuc)] production and the efficiency of Nod factor production by *B. japonicum* strains 532C and USDA110.

2. To examine the response of soybean root hairs to Nod factor under three abiotic stress conditions: low temperature, low pH, and salinity.

3. To determine the effect of application of Nod factor to the root system on soybean powdery mildew disease progression.

4. To evaluate the effect of Nod factor application on the activity of PAL the first enzyme in the phenylpropanoid pathway.

5. To evaluate the effect of Nod factor on fungal growth and development (powdery mildew) in soybean tissues.

6. To determine the effect of low RZT on powdery mildew infection of soybean leaves.

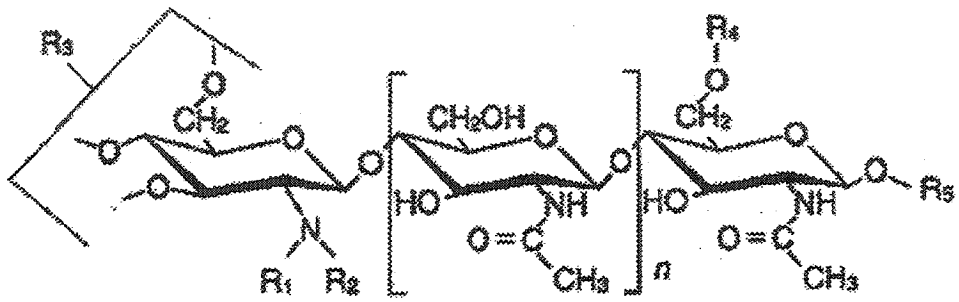


Fig.2.1. The structure of Nod factor. Nod factor from *B. japonicum* denoted as Nod Bj-V ($C_{18:1}$, MeFuc). Bj stands for *B. japonicum*, V indicates the number of *N*-acetylglucosamine residues in a chitin backbone. In the bracket, the length of the acyl chain and degree of unsaturation, and substitutions on the reducing terminal sugar residue are mentioned. The R groups stand for hydrogen (R_1), fatty acyl chain (R_2), hydroxy (R_3, and R_5), MeFuc (R_4) and n = 5 (chitin pentamer) (source: http://www.glycoforum.gr.jp).

Preface to Chapter III

Chapter 3 is comprised of a manuscript prepared by H. Duzan, A. Souleimanov, and D.L. Smith, to be submitted for publication to Soil Biology and Biochemistry. The format has been changed to be consistent within this thesis. All literature cited in this chapter are listed in the reference section at the end of the thesis. All Figures for this chapter are presented at the end of this chapter.

The co-authors in this chapter are as follows: Professor D.L., Smith, and Dr. A. Souleimanov. Their contributions are described in detail in the previous section of contributions of authors.

The *Bradyrhizobium*-soybean symbiosis, is affected by a number of environmental factors, including low RZT, which can be a limiting factor to soybean production in short season areas such as Eastern Canada. Early stages, in particular signal exchange between the two partners, can be affected by suboptimal growth temperature. Previous work in our laboratory addressed the effect of low RZTs on soybean signals from the plant to the rhizobia. In this section, I have shown that suboptimal growth temperatures inhibit the soybean-rhizobia association by inhibiting production of Nod factor, the bacteria-to-plant signals that play a key role in nodule initiation.

Chapter III

Nod Bj-V ($C_{18:1}$, MeFuc) Production by *Bradyrhizobium japonicum* (USDA110, 532C) at Suboptimal Growth Temperatures and its Biological Activity

H. M. Duzan, A. Souleimanov and D.L. Smith*

*Department of Plant Science, Macdonald Campus of McGill University, 21, 111 Lakeshore, Sainte-Anne-de-Bellevue, QC, H9X 3V9, Canada

3.1. Abstract

Nod factor (Lipo-chitooligosaccharides, or LCOs) act as bacteria-to-plant signal molecules that modulate early events of the *Bradyrhizobium japonicum* - soybean symbiosis. Sub-optimal rhizosphere temperatures suppress plant-to-bacteria signaling by decreasing production of flavonoid signals, particularly genistein, resulting in poor nodulation and lower amounts of fixed nitrogen. Addition of appropriate flavonoids to soybean inoculants partially compensates for the impact of low soil temperature early in the growing season, however, the effect on bacteria-to-plant signaling is largely uninvestigated. Thus, the objective of this study was to evaluate the effects of low incubation temperatures on the production of Nod factor (LCO) by *B. japonicum*. Two strains of *B. japonicum*, 532C and USDA110, were tested for ability to synthesize Nod Bj-V ($C_{18:1}$, MeFuc) at three incubation temperatures (15, 17 and 28 °C). The greatest amounts of the major Nod factor, Nod Bj-V ($C_{18:1}$, MeFuc), were produced at 28 °C by both strains. At 15 and 17 °C, the Nod factor production efficiency, per cell, of *B. japonicum* 532C and USDA110 was markedly decreased with the lowest Nod factor concentration per cell occurring at 15 °C. Strain 532C was more efficient at Nod factor production per cell than strain USDA 110 at all temperatures. The biological activity of the extracted Nod factor was unaffected by temperature. This study constitutes the first demonstration of reduced Nod factor production (per cell production) under low temperatures, suggesting another way that lower temperatures inhibit establishment of the soybean N_2 fixing symbiosis.

3.2. Introduction

Rhizobia associate with the roots and stems of many leguminous plants, forming a highly specialized structure, the nodule, where the appropriate bacterial partner resides and biological nitrogen fixation occurs. Nodulation is a multi-step process that requires coordinated communication, through the exchange of specific signaling molecules, between plant and bacterial symbiotic partners. The first step involves the secretion of phenolic compounds, mainly flavonoids, by the plants. These compounds activate the Nod D protein which, in turn, binds to the Nod box and activates transcription of the common *nod* genes in rhizobia, resulting in production of bacterial Nod factor.. Nod factor induces pronounced root hair curling where, presumably, rhizobia is entrapped. This is followed by actual entry of bacterial cells through an invagination of the plasma membrane where rhizobia will be contained. Cortical cells then differentiate, giving rise to nodule primordia. Infection threads continue to grow toward the target cells and once they reach the nodule primordia bacteria, contained inside plasmalemma, will be released into the nodule. Presumably due to an unknown signal from the plant, rhizobia dividing and differentiate into bacteroids, where nitrogen will be fixed and exchanged for nutrients supplied by the plant.(Schultze and Kondorosi, 1998; Hungria and Stacey, 1997).

Flavonoid molecules that act as early nodulation signals between legumes and their rhizobial symbionts vary in type and relative amount from one type of host plant to another. Nodulation and N_2 fixation by legume plants are directly affected by flavonoid concentrations (Appelbaum, 1990). Genistein and daidzein are the primary flavonoids acting as signal molecules between soybean [*Glycine max* Merr. (L.)] and *B. japonicum*

(Kosslak et al., 1987; Sutherland et al., 1990). Quercetin and kaempferol are also known soybean inducers of *B. japonicum*, but have less activity than genistein (Kosslak et al., 1987). In response to signals from the plant, the bacteria synthesize and release lipo-chitooligosaccharides, Nod factor, which trigger root hair curling. Bradyrhizobia are entrapped in the root hair curl and this allows an intimate association with the host plant leading to infection thread formation and eventual nodule differentiation (Schultze and Kondorosi, 1998; Lhuissier et al., 2001).

All Nod factors have a similar basic structure, being composed of a linear chain of ß-1,4-linked *N*-acetylglucosamines linked to an acyl side chain on the non-reducing end and a variety of substitutions on the reducing end (Mergaert et al., 1997; Hungria and Stacey, 1997). For example, the most abundant Nod factor produced by *Bradyrhizobium japonicum* USDA110 is Nod Bj-V ($C_{18:1}$, MeFuc). Several other less abundant lipo-chitooligosaccharides, have been characterized from *B. japonicum* strain USDA135 (Sanjuan et al., 1992; Spaink et al., 1992). Nod Bj-V ($C_{18:1}$, MeFuc), was also extracted and purified from *Bradyrhizobium japonicum* 532C (Souleimanov et al., 2002)

Purified or chemically synthesized Nod factor can elicit various responses from legume plants. These responses include root-hair curling, root-hair deformation, formation of pre-infection threads, depolarization of plasma membranes, cortical cell divisions, induction of some early nodulation genes, the formation of thick short roots, and, in some cases, the formation of structures resembling fully formed nodules (van Brussel et al., 1992; Schultze and Kondorosi, 1998). Nod factors are effective at extremely low concentration, 10^{-15} M or 1 ng per spot inoculation were able to trigger root

hair deformation and nodule structure development (Stokkermans and Peters, 1994; Stokkermans et al., 1995).

Soybean growth, when dependent on the rhizobia-legume symbiosis, is affected by a number of environmental factor including low root zone temperature (RZT), which can be a limiting factor to soybean production in short season areas such as Eastern Canada. Temperature is considered to be a major limiting factor for soybean growth in cool areas with relatively short growing seasons (Whigham and Minor, 1978). Early studies showed that the 25 to 30 °C range of root temperatures is optimal for soybean N_2 fixation (Jones and Tisdale, 1921). Low soil temperature is an important factor in symbiotic effectiveness and yield of soybean (Lie, 1981; Lindemann and Ham, 1979). The effect of a given temperature on nodulation and N_2 fixation varies with bacterial strain and cultivar (Gibson, 1971; Lindemann and Ham, 1979). Selection of host plants and bacterial strains adapted to prevailing conditions will minimize adverse environmental effects on nodulation and N_2 fixation (Gibson, 1971). The adaptation of plants and their symbiotic partners was demonstrated under arctic conditions (Ek-Jander and Fahraeus, 1971; Bordeleau and Prevost, 1994). Sensitivity of the host plant to low temperature can be modified by the strain of bacterial symbiont used (Lie, 1981). Dart et al. (1976) recommended that *B. japonicum* strains be selected at soil temperatures likely to be encountered during growth.

Because soybean requires temperatures in the 25 to 30 °C range for optimum N_2 fixation and yield low seasonal temperatures (generally May and September) restrict the growth of N_2 fixing soybean in eastern Canada (Lynch and Smith, 1993). All stages in

establishment of the soybean-*Bradyrhizobium* symbiosis (root hair curling, infection thread formation and penetration, nodule development and function) are inhibited by suboptimal RZTs; however, infection processes are the most sensitive steps (Zhang and Smith, 1994). Studies of the effects of suboptimal RZTs on soybean concluded that these conditions decrease N fixation activity by the nitrogenase enzyme complex (Layzell et al., 1984) and suppress or delay root infection and nodulation (Walsh and Layzell, 1986). Between 25 and 17 °C the time from inoculation and the onset of N_2 fixation was delayed by 2 days for each degree decrease in temperature, whereas RZTs between 17 and 15 °C were more strongly inhibitory, and each degree decrease delayed the onset of N_2 fixation by about one week (Zhang et al., 1995). In field bean (*Phaseolus vulgaris* L.), the period from sowing to induction of nitrogenase activity was about 3 times as long at 10 °C (45 days) as at 18 °C (14 days); this difference was attributed to a greatly prolonged pre-infection period (24 days at 10 °C, 8 days at 18 °C) and slower differentiation of nodules at the lower temperature. Under optimal conditions only 24 h are required for the bacteria to initiate infection threads in field bean (Fyson and Sprent, 1982). In addition, the fatty acid composition of free-living *R. leguminosarum* bv. *viciae* strains CBh5 and CBp7 were markedly affected by low temperatures (Theberge et al., 1996).

A lesser genistein content in soybean roots was reported at lower RZTs, in addition, bacterial *nod* gene induction (*nodY-lacZ* fusion of *B. japonicum* USDA110) was inhibited at low incubation temperatures, adding a higher genistein concentration overcomes this inhibitory effect (Zhang and Smith, 1996; Pan and Smith, 1998). Nod factor production/excretion by *Rhizobium leguminosarum* bv. *trifolii* was disrupted at low incubation temperatures, and this was attributed to effects on transcription, translation, or

post-translation events involved in Nod factor production (McKay and Djordjevic, 1993). Taken together data of previous studies show that low RZTs suppress signaling between symbiotic partners. Addition of appropriate flavonoids (the plant-to-bacteria signals) to soybean inoculants partially compensates for the impact of low RZTs. Low temperature also affects the growth of rhizobial cells and, in the end, the direct measure of effects specifically on Nod factor production must be Nod factor produced per cell, or Nod factor production efficiency, however, the effect of low RZTs on the efficiency of signaling from the bacteria (lipo-chiooligosaccharides) is largely uninvestigated. Thus the objective of this study is to evaluate the effects of low growth temperatures on the production of the major Nod factor [Nod Bj-V ($C_{18:1}$, MeFuc)] produced by cells of *B. japonicum* strains (532C and USDA110).

3.3. Materials and Methods

3.3.1. Bacterial strains

Two strains of *B. japonicum*, 532C and USDA110, were obtained from Liphatech Inc. (Milwaukee, WI, USA) and tested for Nod factor production at three incubation temperatures (15, 17, and 28 °C). These strains were selected as they are, or have been, widely used in commercial *B. japonicum* inoculants sold in Canada. Strain 532C is currently used in most Canadian inoculants and strain USDA110 was used in Canadian inoculants and is still used in American inoculants.

3.3.2. Bacterial culture and growth conditions

This experiment was conducted to study the effect of three growth temperatures (15, 17, and 28 °C) on Nod factor production. Two strains were utilized: *B. japonicum* 532C and USDA110. The treatments consisted of factorial combinations of the two strains and the three growth temperatures. Each treatment was replicated five times. In order to observe clear separation and elution of the targeted Nod factor peak, isolated and quantified by HPLC, the *B. japonicum* cells were grown in Bergersen minimal (BM) medium and prepared as described by Spaink et al. (1992).

The cultures were grown in 200 mL of Bergersen minimal (BM) medium at 28 °C, originating from starter cultures of the same OD (A_{620} 0.08) values for the two strains tested. On the sixth day of culture, five replicates of each strain were transferred to 17 °C, and five to 15 °C incubation temperatures. Five were also left at 28 °C. Throughout the work the cultures were held in glass flasks and shaken at 150 rpm on an incubator orbital shaker (model 4580, refrigerated console, Forma Scientific Inc. USA). After 24 h of acclimatization to the new growth temperatures, 5 μM genistein were added to each replicate (flask). Nod factor extraction was performed after five days. It is known that 5 μM genistein provides for maximum Nod factor production, on a whole culture basis, at optimal temperatures (Kosslak et al., 1987; Zhang and Smith, 1995; Zhang et al., 1996); concentration was utilized in order to determine the effects of temperature on Nod factor production under standard conditions.

3.3.3. Population density assay

Nod factor was extracted and purified form bacterial strains incubated under suboptimal growth temperatures; therefore, there was a need to determine whether reductions in Nod factor production, due to low temperature, at the culture level were because of reduced production per cell or reduced cell numbers or both. Therefore, a cell density assay was conducted for each bacterial culture of each treatment and replicate. Serial dilution and spread-plate methods (Somasegaran and Hoben, 1994) were performed to determine the number of viable cells in the cultures. In order to achieve a countable cell number (30 to 300 cells mL^{-1}) a range of 10^{-1} to 10^{-8} culture dilutions was prepared. Four sub-replicates of 0.1 mL from each dilution per replicate per treatment, for a total of 4 x 5 x 3 x 2 = 120 treatments, were plated on yeast extract mannitol agar (Vincent, 1970) and incubated at 28 °C. Numbers of colonies were counted after 7 to 9 days of incubation. Number of colony forming units / mL (CFU/mL) was determined with the following formula:

CFU/ml = $\sum$ No. of colonies per plate / No. of plates counted *1/mL of aliquot *1/decimal dilution

3.3.4. Nod factor extraction and purification

The 200 mL cultures of each treatment/replicate were extracted with 40% HPLC-grade 1-butanol, followed by shaking the mixture for 5 to 10 minutes, then allowing the two phases to separate for 24 h. The organic phase (butanol layer) was collected and evaporated at 80 °C in a Yamato RE500 Rotary evaporator (Yamato Scientific American Inc., NY, USA). The extract was dissolved in 1 to 2 mL of 18% of acetonitrile and stored

in the dark in glass tubes at 4 °C for 24 h. Samples were centrifuged for 10 minutes at 12 g, and the supernatant was collected for HPLC analysis.

Two hundred μL of the Nod factor extract were injected into a Waters HPLC system (Waters Associations Inc., Milford, MA) consisting of a model 712 WISP, two model 510 pumps and a model 441 UV detector operating at 214 nm. Separation was carried out with a Vydac C18 reversed-phase column (5 μm, 46 X 250 mm, Vydac, USA). A program of gradients of acetonitrile and water was used to elute Nod factor from the column: 18% acetonitrile (10 min), from 18 to 60% acetonitrile (20 min), from 60 to 100% acetonitrile (5 min). The Nod Bj-V ($C_{18:1}$, MeFuc) was eluted at 30.to 71 min of HPLC run time (Fig. 3.2.). The elution time was comparable to the elution time of a standard (a gift from Prof. G. Stacey, Center for Legume Research, University of Tennessee, Knoxville, USA).

3.3.5. The biological activity of Nod Bj-V ($C_{18:1}$, MeFuc)

The Nod factor was isolated by HPLC. There was a concern that different temperature regimes could cause the production of non-Nod factor compounds that co-elute with the Nod factor of interest. Thus, we bioassayed the material collected from the HPLC, and assumed to be the major Nod factor produced by *B. japonicum*. Nod Bj-V ($C_{18:1}$, MeFuc) was extracted and purified from each bacterial culture incubated, at a range of growth temperatures.

For root hair deformation assay, soybean (OAC Bayfield) seeds were surface sterilized in 2% sodium hypochlorite for 2 minutes and washed thoroughly, several times, with distilled, sterilized water. Two seeds were placed on Petri plates containing 1.5 % water agar, and incubated in the dark at room temperature. After 5 to 7 days, lateral roots of similar length and showing abundant root hairs were aseptically excised and placed on slides. Three slides per treatment were prepared; each contained three lateral roots, for a total of 9 observations per Nod factor extracted from each incubation temperature. Two concentrations, 0 (distilled water control) and10^{-6} M were applied to lateral roots as 10^{-6} M is known to trigger root hair deformation (Prithiviraj et al., 2000; Duzan et al., chapter four). Slides were kept in a closed moist chamber and incubated for 24 h at 25 °C in the dark. Roots were fixed with staining solution (Prithiviraj et al., 2000). Fields of root hair deformations were observed using light microscopy (Jenalumar, Jena Instruments Ltd, Germany) in the zone where susceptible root hairs occur, [generally zones I and II where active root hair elongation is occurring, but not zone III where elongation is finished, about 1 to 2 (Prithiviraj et al., 2000) mm above the root tip for domestic soybean] until all root hairs in the susceptible zone were observed (Stokkermans et al., 1995; Prithiviraj et al., 2000). The entire experiments were conducted twice with similar results in both instances.

3. 4. Results

3.4.1. The effect of incubation temperature on per culture Nod factor production

The greatest amounts of the major Nod factor (Sanjuan et al., 1992; Souleimanov et al., 2002), Nod Bj-V ($C_{18:1}$, MeFuc), were produced by the two strains at 28 °C. However, the concentration varied between the tested strains (Fig. 3.1. A). At 5 μM genistein, and at 28 °C Nod factor production by *B. japonicum* 532C was much greater than by USDA110. When incubation temperature dropped to 17 °C, a large decrease in Nod factor concentration was observed, as compared with concentrations detected at 28 °C, an optimal growth temperature. The lowest level of Nod factor production occurred at 15 °C for both strains (Fig. 3.1. A).

At sub-optimal incubation temperatures (15 and 17 °C) the growth rates of strains 532C and USDA110 in the culture medium were markedly decreased. Cell density was different between the two tested strains. At 17 and 28 °C, USDA110 had more CFU / mL than 532C, while, at 15 °C USDA110 had fewer CFU/mL than 532C (Fig. 3.1. B).

The greatest production of Nod factor per bacterial cell was observed at the optimal growth temperature (28 °C) for both strains. There was a direct relationship between growth temperature and efficiency of bacterial cells for Nod factor production, with the lowest efficiency occurring at 15 °C. In general, 532C was more efficient than USDA110 in Nod factor production at the three tested growth temperatures (Fig.3.1. C).

3.4.2. Effect of temperatures on the biological activity of Nod factor

Nod Bj-V ($C_{18:1}$, MeFuc) from 532C and USDA110, produced by all treatments, exhibited bioactivity on soybean root hairs. Three types of root hair deformations were observed in the root hairs of the susceptible zones: wiggling, bulging, and curling. In general, Nod factor from all the treatments manifested activity (root hair deformation) levels that were not different at 10^{-6} M when applied to soybean root hairs. Control solutions, without Nod factor, showed none of the typical Nod factor induced HAD activity (Fig. 3.3. B to F).

3.5. Discussion

Soybean-bradrhizobia symbiosis establishment can be affected by a number of abiotic stress conditions, such as low temperature, which can be a major limiting factor for soybean nodulation and N_2 fixation (Whigham and Minor, 1978). Previous studies addressed the inhibitory effect of low RZTs on "inter-organismal signal exchange", particularly the inhibition of genistein accumulation (the plant-to-bacteria signals) in soybean roots (Zhang and Smith, 1996; Pan and Smith, 1998). Here we present evidence regarding the effect of suboptimal growth temperature on production of Nod factor, the bacteria-to-plant signal, by two strains of *B. japonicum.*

When the growth temperature was reduced to 17 and 15 °C lower Nod factor concentrations resulted for both of the tested strains. This is in agreement with the previously described effect of low incubation temperatures (18 °C) on the detected whole

culture level of *Rhizobium leguminosarum* bv. *trifolii* Nod factor production (McKay and Djordjevic, 1993). The progressive reduction in Nod factor production as temperature decreased from 28 to 15 °C, for both strains, does not necessarily indicate direct effects of the growth temperature on Nod factor production; it is possible that the low growth temperature disrupts Nod factor excretion rather than Nod factor production, as reported by McKay and Djordjevic (1993); this was not tested in the current study. Furthermore, *nod* gene expression in *B. japonicum* USDA110 is inhibited by low incubation temperatures (Zhang et al., 1996), hence, it is reasonable, although previously undemonstrated, to observe lower Nod factor production by at least strain USDA110 grown under suboptimal growth temperatures.

As incubation temperature decreased, so did cell density, for both strains, however, the cell density of USDA110 was higher, at 28 and 17 °C, but lower at 15 °C, than 532C. Conversely, 532C produced more Nod factor than USDA110 at all temperatures. Perhaps at the lowest incubation temperature (15 °C) USDA110 is more sensitive and thus rate of cell growth and metabolism is highly affected by low temperature, which would have resulted in the lowest production of Nod factor. The better growth of 532C than USDA110, at 15 °C may contribute the better suitability of the former for use in commercial inoculants in Canada, where spring soil conditions are generally cool. Perhaps it is not surprising that low incubation temperatures affected the gross rate of Nod factor production and rate per cell. Zhang et al. (1996) previously reported the inhibitory effect of low incubation temperature on growth rate and efficiency of bacterial cell in *nod* gene induction for USDA110. Similar results, in general, were reported by (Zhang et al., 2002), though, we can not specifically compare their findings

with data reported in the current study for four reasons: 1) Zhang et al. (2002) monitored bacterial growth rate based on OD observations, 2) bacterial cultures were induced with suboptimal levels of genistein (0.1, 1.0 μM), 3) yeast extract mannitol broth (YEM) medium was used to grow the culture, and 4) the range of temperature tested for Nod factor production was not the same (only 17 and 25 °C). It should also be noted, that repression of *nod* gene induction has been reported at high *B. japonicum* population densities, mediated by a quorum signal and governed by the regulatory NodD2 expression (Loh et al., 2001), which can lead to lesser Nod factor production at higher population densities. Differential sensitivities to quorum sensing signal, or differential production of this signal, could cause differences in Nod factor production at high population densities.

Both, the rate of growth of bacterial strains and the efficiency of Nod factor production (amount of Nod factor produced per cell) were reduced by low incubation temperature, and when production at lower temperatures were expressed as a percentage of production at an optimal incubation temperature (28 °C) the decreased concentration of Nod Bj-V ($C_{18:1}$, MeFuc) produced by strain USDA110 at 15 °C was primarily due to lower numbers of bacterial cells (decreased by 80%) rather than efficiency (decreased by 69%), while, efficiency of 532C in Bj-V ($C_{18:1}$, MeFuc) production was more affected (decreased by 70%) than the rate of growth (decreased by 38 %). At 17 °C a similar pattern was observed for the two strains (USDA110, 532C), where the efficiencies were more affected (decreased by 55 and 63%) than the growth rate (30 and 29 %) respectively. Per cell production of Nod factor was more affected by low temperatures for strain USDA110 than strain 532C. Differential rates of Nod factor production among strains of *R. leguminosarum* bv. trifolii at suboptimal growth temperature was previously

reported (McKay and Djodjevic, 1993). Nod factor production, both per cell and on a whole culture basis, was greater for 532C than USDA110 at all three growth temperatures; perhaps this is another mechanism contributing to the superior performance of strain 532C over a range of temperatures, contributing to its widespread use under Canadian conditions (Hume and Shelp, 1990; Lynch and Smith, 1993). Suboptimal growth temperature has been shown to reduce the amount of genistein in soybean roots and inhibit the expression of *nod* genes, resulting in delayed nodulation and onset of N_2 fixation (Zhang and Smith, 1994; Zhang et al., 1996). In the present study, low growth temperatures have been shown to inhibit the production of Nod factor, the bacteria-to-plant signal.

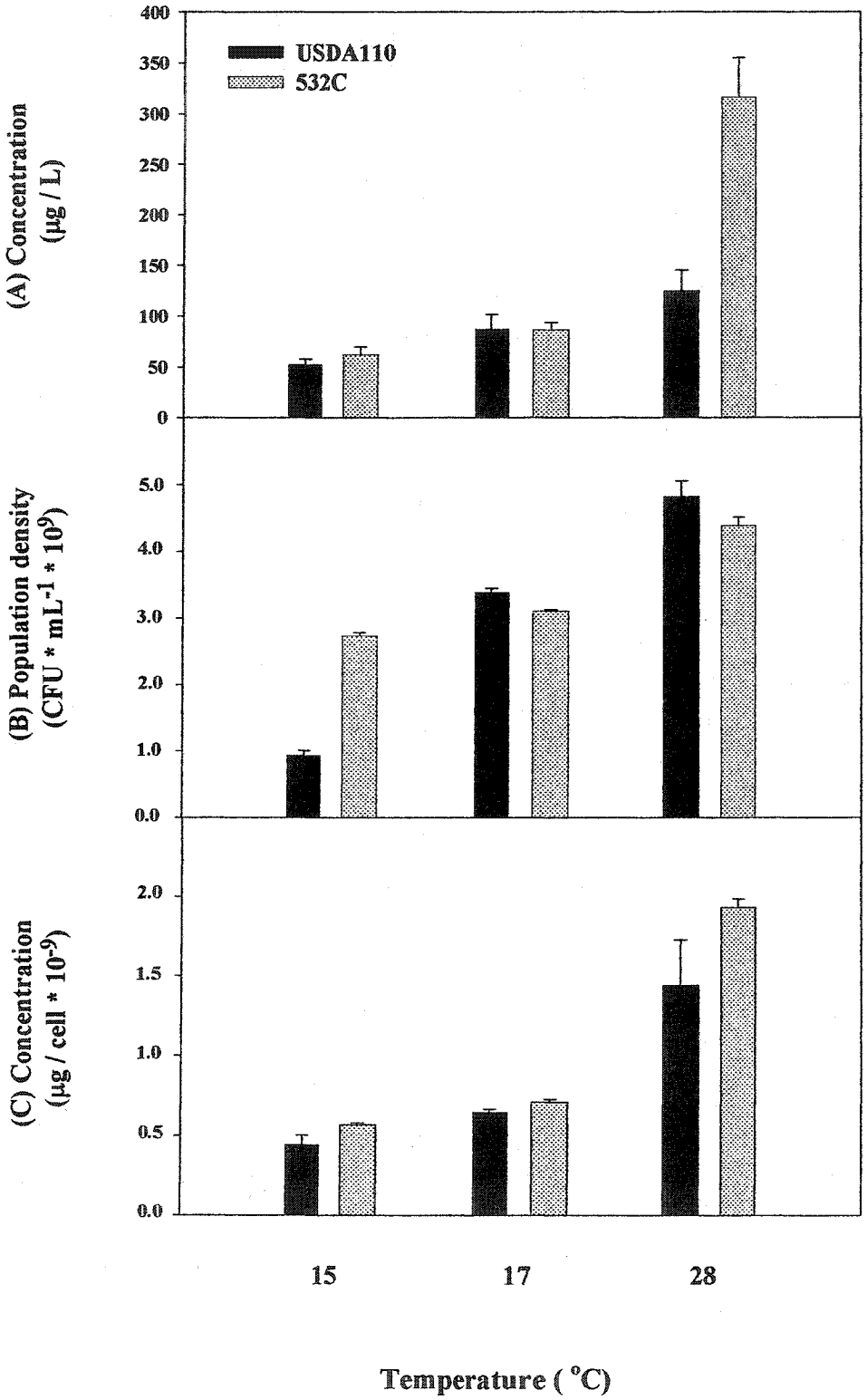
The earliest visible response of root hairs to Nod factor application is root hair deformation (Lhuissier et al., 2001). Here we conducted qualitative tests of the growth conditions and biological activity relationships of purified Nod factor. The data allows us to conclude that Nod Bj-V ($C_{18:1}$, MeFuc) extracted from *B. japonicum* strains (USDA110, 532C) maintained its bioactivity, ability to induce soybean root hair deformations, at all temperatures. Recently, the effect of low incubation temperature on the perception of Nod factor by soybean root hairs was addressed (Duzan et al., chapter four).

In conclusion, these experiments showed that 1) low growth temperature inhibits the biosynthesis/excretion of Nod Bj-V ($C_{18:1}$, MeFuc) production by two strains commonly used in commercial inoculants, 2) the growth rates were reduced by low incubation temperature, 3) low growth temperature affects the response of the

microsymbiont to *nod* gene inducers, 4) all Nod factor extracts caused root hair deformations that were not different when the Nod factor solutions were tested at the same concentration, 5) strain 532C was more efficient in Nod factor production per cell, over the range of tested growth temperatures, than USDA110.

Further studies should investigate the impact of low temperature on: Nod factor biosynthesis, excretion and chemical structure, and their influences on the ability of the microsymbiont to produce Nod factor at low temperature, and the perception of Nod factor by the macrosymbiont. We evaluated Nod factor production under unfavorable growth conditions using 5 μM genistein, the concentration that induced the highest level of *nod* gene expression at 25 °C (Zhang et al., 1996). Therefore, it is reasonable to hypothesize that induction of *nod* genes with higher concentrations of genistein potentially overcomes the stressful effects of low temperature on metabolites involved in Nod factor production; however, this was not tested in the work reported here.

Fig. 3.1. The effect of incubation temperature on Nod Bj-V ($C_{18:1}$, MeFuc) production in two strains of *B. japonicum* (USDA110 and 532C). Each value is plotted as the mean ± S.E. (n = 5).


USDA110
532C
(A) Concentration (μg / L)
400
350
300
250
200
150
100
50
0
(B) Population density (CFU * mL-1 * 10^9)
5.0
4.0
3.0
2.0
1.0
0.0
(C) Concentration (μg / cell * 10^-9)
2.0
1.5
1.0
0.5
0.0
15
17
28
Temperature (°C)

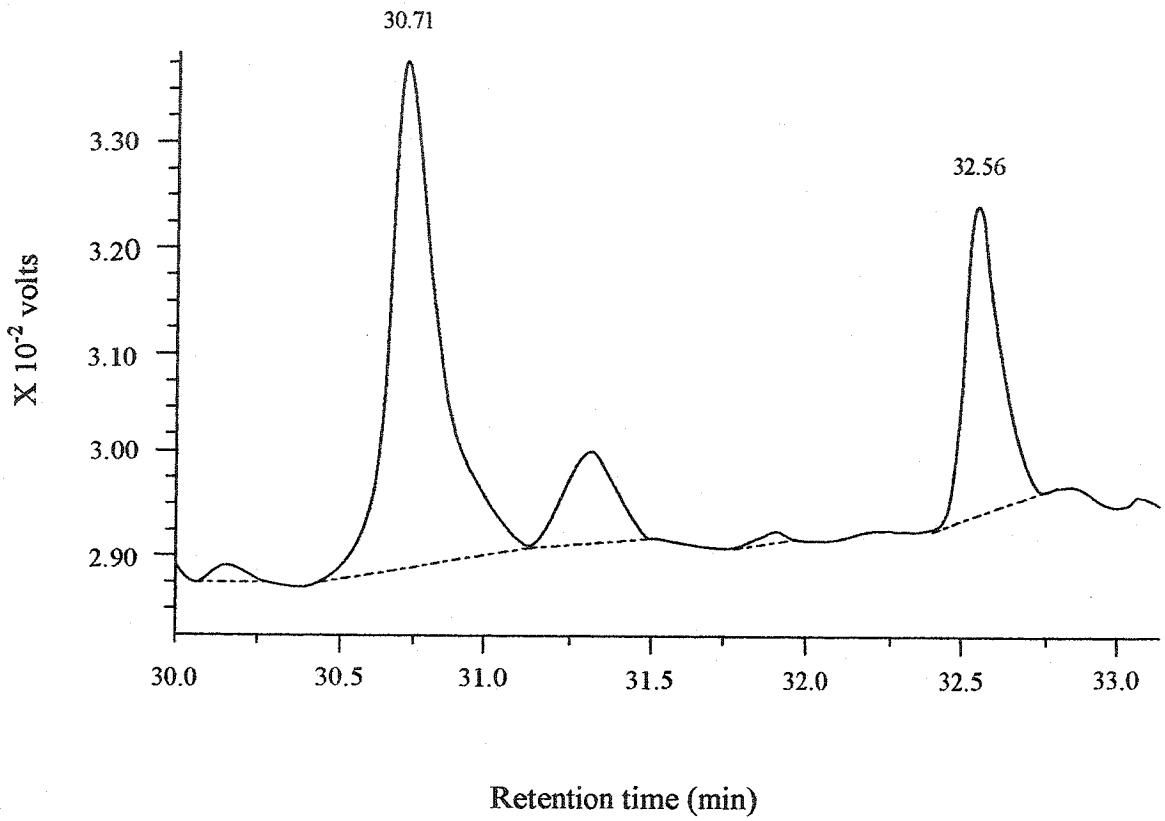
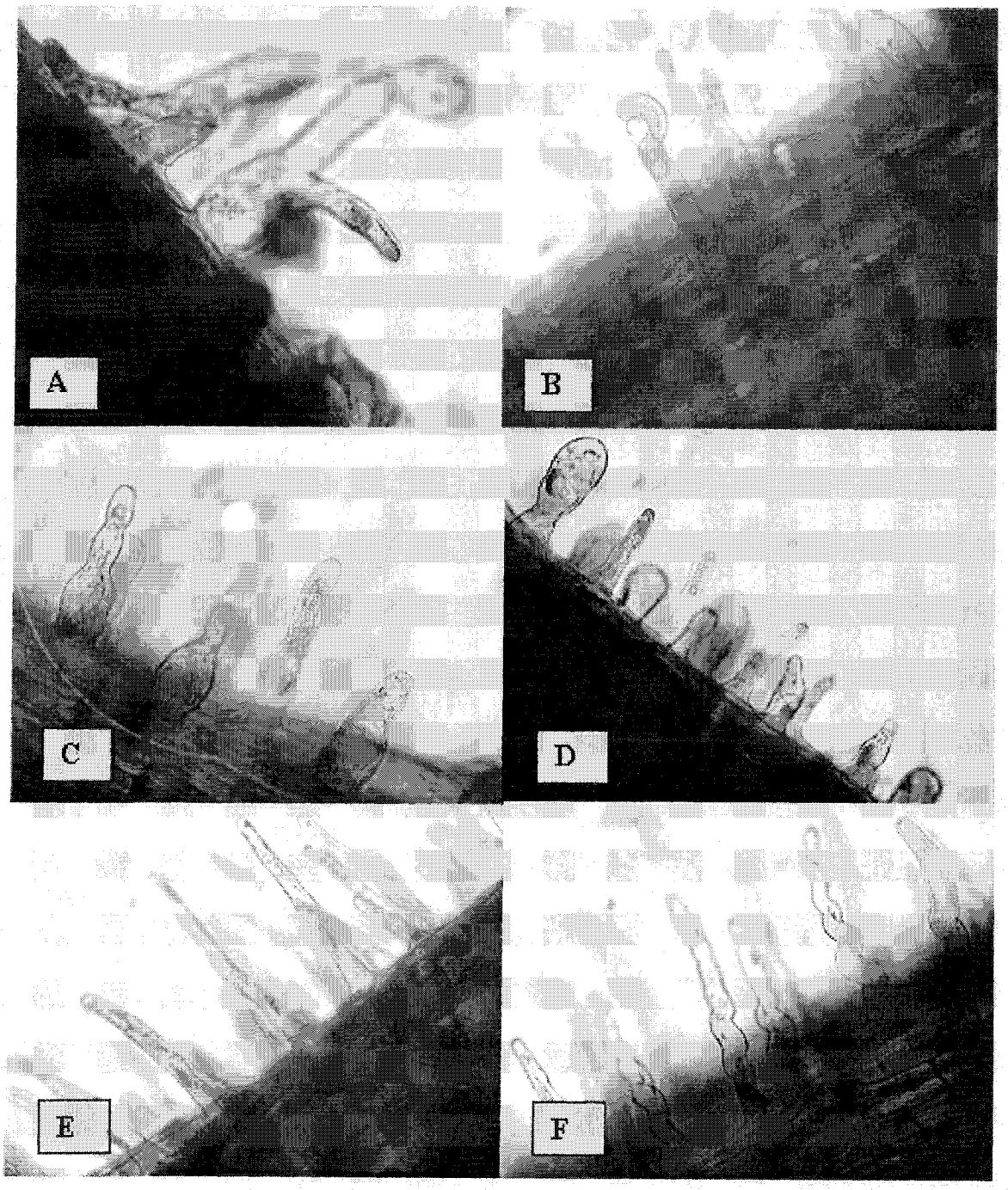


Fig. 3.2. HPLC profile of *n*-butanol extract from *Bradyrhizobium japonicum* (USDA110, 532C). Nod Bj-V ($C_{18:1}$, MeFuc) with retention time 30.71 min, Nod Bj-V (Ac, $C_{16:0}$, MeFuc) with retention time 32.56 min.

Fig. 3.3. Types of root hair deformations manifested by Nod factor treatments. (A) Root hair branching (magnification = 615). (B) Root hair curling (magnification = 308). (C and D) Root hair bulging (magnification = 288). (E) Untreated root hairs (control, magnification = 462). (F) Root hair wiggling (magnification = 384).


A
B
C
D
E
F

Preface to Chapter IV

Chapter 4 is comprised of a manuscript prepared by H. Duzan, Dr. X. Zhou, Dr. A. Souleimanov, and D.L. Smith, for submission to Journal of Experimental Botany. The format has been changed to be consistent within this thesis. All literature cited in this chapter are listed in the reference section at the end of the thesis. All Figures for this chapter are presented at the end of this chapter.

The co-authors to this chapter are Dr. D.L. Smith, Dr. X. Zhou, and Dr. A. Souleimanov. Details of their contributions were described in the section of contributions of authors.

In the previous chapter we addressed the effect of suboptimal growth temperature on Nod factor production by *B. japonicum* and found that this contributes to the low RZT reduction of soybean nodulation. In this chapter we investigated the effect of three abiotic stresses (suboptimal temperature, low pH, and salinity) on the biological activity of Nod factor and their perception by soybean root hairs.

Chapter IV

Recognition of *Bradyrhizobium japonicum* Nod factor by Soybean [*Glycine max* (L.) Merr.] Root Hairs under Abiotic Stress Conditions

H. M. Duzan, X. Zhou., A. Souleimanov., and D.L. Smith*

*Department of Plant Science, Macdonald Campus of McGill University, 21, 111 Lakeshore, Sainte-Anne-de-Bellevue, QC, H9X 3V9, Canada

4.1. Abstract

Suboptimal growth conditions, such as low rhizosphere temperature, high salinity, and low pH can negatively affect early stages of the rhizobia-legume symbioses, resulting in poor nodulation and lower amounts of fixed nitrogen. Nod factor act as bacteria-to-plant signals that modulate early events of the *Bradyrhizobium japonicum* - soybean [*Glycine max* (L.) Merr.] symbiosis. Addition of Nod factor to rhizobia-legume systems partially compensates for abiotic stresses in the early growing season; however, the effect on bacteria-to-plant signaling, particularly, root hair deformations is unknown. Thus, the objective of this study is to evaluate the perception of Nod factor by root hairs under three stress conditions, low temperature, low pH, and high salinity. Three experiments were conducted using a 1:1 ratio of Nod Bj-V ($C_{18:1}$, MeFuc) and Nod Bj-V (Ac, $C_{16:0}$, MeFuc). Nod factor induced four types of deformations, wiggling, bulging, curling, and branching. Under optimal experimental conditions the response of root hairs to the three tested levels of Nod factor (10^{-6}, 10^{-8}, and 10^{-10} M) was dose dependant. There was no interaction between low temperature and Nod factor concentration, or low pH and Nod factor concentration. Root hair deformation (HAD) decreased with temperature over the range tested (25, 17, and 15 °C). Low pH, high salinity and low temperature all reduced HAD caused by Nod factor. Salinity strongly inhibited HAD responses to Nod factor; there was no increase in HAD as Nod factor concentration increased when the root hairs were exposed to saline conditions, but, this relationship was seen in control (no salinity) plants, resulting in a Nod factor concentration by salinity concentration interaction.

4.2. Introduction

Successful rhizobia-legume symbioses depend on efficient plant root nodulation and subsequent N_2 fixation. During the early stages of infection and nodule organogenesis, bacteria attach to the plant roots in response to an attractant exuded by the roots, mainly flavonoids, which activates bacterial NodD protein, causing transcription of the bacterial *nod* genes. Expression of the *nod* genes results in synthesis of bacteria-to-plant signal molecules, Nod factor; these compounds are composed of tri- to penta-chitin backbones and possess an *N*-acyl group at the non-reducing end and a variety of substitutions at locations along the chitin backbone. Differences among Nod factor specify compatibility between macrosymbiont and microsymbiont species (Schultze and Kondorosi, 1998). Application of appropriate Nod factor to soybean (*Glycine max* (L.) Merr.) roots triggers responses such as root hair deformation, and can cause the initiation of nodule primordia or the formation of complete nodule structures (Carlson et al., 1993; Stokkermans et al., 1995). It is now known that the root hair deformation elicited by Nod factor involves signal transduction through phospholipase C and D (Hartog et al., 2001). Recently, the role of phospholipase D in root hair morphogenesis was confirmed in *Arabidopsis thaliana* (Ohashi et al, 2003).

Abiotic stress factor, such as salinity, low pH, and low root zone temperature can cause poor nodulation in the presence of otherwise compatible symbionts. Early events in the symbiosis such as, signal production and excretion, rhizobial attachment, root hair curling, infection thread formation, and nodule initiation, are particularly sensitive to

these stresses (Tu, 1981; Mckay and Djordjevic, 1993; Zhang and Smith, 1996; Hungria and Stacey, 1997; Hungria and Vargas, 2000; Duzan et al., chapter three).

Salinity stress is a major environmental constraint on rhizobia-legume associations, affecting their capacity for nodulation and N_2 fixation. The development of shoot; root, and nodule dry weights by faba bean (*Vicia faba L.)* were decreased by 100 mM NaCl (Cordovilla et al., 1999). A 50% reduction in soybean nodule number and weight was associated with extreme sensitivity of nodule initiation to salinity, and this was attributed to limited root growth and decreases in the proportion of infected root hairs (Singleton and Bohlool, 1984; Saadallah et al., 2001). Further, reduction in soybean nodule number by 10.0 dS m^{-1} (195.9 mM NaCl) resulted in decreased N_2 fixed per nodule (Elsheikh and Wood, 1995). At 1.2 % (205.6 mM) NaCl, soybean nodulation was completely eliminated (Tu, 1981).

The growth and survival of free living rhizobial cells can also be affected. Generally, rhizobia are less affected (growth and survival) by salt stress than their plant host (reviewed by Grag and Gupta, 2000). *Rhizobium* strains are more salt-tolerant than *Bradyrhizobium* strains and differential responses among *Bradyrhizobium sp.* strains were reported to increasing concentrations of NaCl (50 – 400 mM) (Elsheikh, 1998).

The sensitivity of early stages, in particular the mutual exchange of signal molecules between nitrogen fixing microorganism and higher plant, to low pH was also addressed (McKay and Djordjevic, 1993; Hungria and Stacey, 1997). Excretion of *nod* gene inducers by subterranean clover (*Trifolium subterraneam* L.) roots was inhibited at

pHs below 5 (Richardson et al., 1988a); and, further, limited induction of *nod* genes in three tested strains of *Rhizobium leguminosarum* biovar *trifolii* under low pH conditions was reported (Richardson et al. 1988b). Poor expression of the common *nod genes* (*nod ABC*) was associated with poor root hair curling (Djordjevic et al., 1985). At pH 4.8 reduced concentrations of *nod* gene encoded metabolites were measured, and this was attributed to inhibition of Nod factor excretion rather than production, with differences occurring among tested strains (McKay and Djordjevic, 1993). Furthermore, nodule initiation, number, and dry weight for soybean plants were negatively affected by low pH (Alva et al., 1987); similar results have been reported for bean (*Phaseolus vulgaris*), where addition of *nod*-gene inducers (eg. genistein) potentially overcomes the stress imposed by low pH growth conditions (Hungria and Stacey, 1997). Rhizobia growth and survival can also be affected by low pH growth conditions. At pH 4.0, *B. japonicum* A1017 exhibited a higher sensitivity to low pH-stress than *R. fredii* P220. This was attributed to the ability of the fast growing bacteria to maintain higher contents of homospermidine and cellular Mg^{2+} (Fujihara and Yoneyama, 1993).

The optimum temperature for soybean nodulation and N_2 fixation is the 25-30 °C range (Jones and Tisdale, 1921). In eastern Canada, low seasonal temperatures (usually May to September) restrict the growth of N_2 fixing soybean (Lynch and Smith, 1993), affecting all stages of symbiosis establishment including signal excretion, root hair curling, infection thread formation and penetration, and nodule development and function, with the infection processes being the most sensitive steps (Walsh and Layzell, 1986; Zhang and Smith, 1994). Low RZTs also reduce the N_2 fixation activity of the nitrogenase enzyme complex (Layzell et al., 1984).

When the RZT was between 25 and 17 °C, the time between inoculation and the onset of N_2 fixation was delayed by 2 days for each degree decrease in temperature, whereas between 17 and 15 °C each degree delayed the onset of N_2 fixation by about 1 week (Zhang et al., 1995). Similar results have been observed in field bean, presumably due to a prolonged pre-infection period (24 days at 10 °C, 8 days at 18 °C) as well as slower differentiation of nodules at the lower temperature (Fyson and Sprent, 1982). Under optimal conditions only 24 h are required for the initiation of infection threads (Fyson and Sprent, 1982).

Suboptimal soil temperature inhibits the excretion of genistein (soybean to *Bradyrhizobium* signal) resulting in less *nod* gene expression (*nody-lacZ* fusion of *B. japonicum* USDA110), and less Nod Bj-V ($C_{18:1}$, MeFuc) production as temperature declined from 17 to 15 °C (Zhang and Smith, 1996; Pan and Smith, 1998; Duzan et al., chapter three). Similar results were reported for *Rhizobium leguminosarum* bv. *trifolii* Nod factor (McKay and Djordjevic, 1993).

During the early stages of symbiosis, the first visual response induced by Nod factor in the presence of rhizobia is root hair curling. Under optimal growth conditions, addition of purified Nod factor to legume root system induces diverse types of root hair deformation, including, wiggling, curling, bulging, and branching (Heidstra et al., 1994; Cullimore et al., 2001; Kelly and Irving, 2002). However, how this phenomenon is affected by abiotic stress factors is not known. Thus, we evaluated the biological activity of an equivalent ratio of Nod Bj-V ($C_{18:1}$, MeFuc) and Nod Bj-V (Ac, $C_{16:0}$, MeFuc)

extracted and purified from *B. japonicum* and added to soybean [*Glycine max* (L.) Merr.] roots under three abiotic stress conditions: salinity, low pH and low temperature.

4.3. Materials and Methods

4.3.1. Bacterial culture

Bradyrhizobium japonicum 532C was obtained from Liphatech Inc. (Milwalkee, WI, USA). This strain was selected as it is widely used in commercial *B. japonicum* inoculants in Canada. The culture was grown at 28 °C in 250 mL yeast mannitol broth (YEM) with shaking at 150 rpm for 4 to 6 days on an incubator orbital shaker (model 4580, refrigerated console, Forma Scientific Inc. USA), and thereafter subcultured into 2000 mL (25 mL per 2000 mL of YEM medium). After 7 days of subculture (OD_{620} of 0.4 to.0.6), bacterial cultures were induced for Nod factor synthesis and production through the addition of 5 µM genistein and incubated for an additional 48-96 h.

4.3.2. Nod factor extraction and purification

The resulting 2 L of culture was extracted with 40% HPLC-grade 1-butanol by shaking the mixture for 5-10 minutes and then allowing the two phases to separate for 24 h. The organic phase (butanol layer) was collected and evaporated at 80 °C in a Yamato RE500 Rotary evaporator (Yamato Scientific American Inc., NY, USA). The final volume, 2 to 3 mL, was dissolved in 4 mL of 18% of acetonitrile and stored in the dark in glass tubes,

at 4 °C for 24 h. Samples were centrifuged for 10 minutes at 12,000 g, and the supernatant was collected for HPLC analysis.

For analytical purposes, during the first run, two hundred µL of the Nod factor extract were injected into a Waters HPLC system (Waters Associates Inc., Milford, MA) consisting of a model 712 WISP, two model 510 pumps and a model 441 UV detector operating at 214 nm. Separation was carried out with a Vydac C18 reversed-phase column (5 µm, 46 X 250 mm, Vydac, USA). To elute Nod factor from the column, a gradient of acetonitrile and water was used: 18% acetonitrile (10 min), a linear gradient from 18 to 60% acetonitrile (20 min), a linear gradient from 60% to 100% acetonitrile (5 min). A chromatographic peak with retention time 31.71 min was identified as Nod Bj-V ($C_{18:1}$, MeFuc) by comparing its retention time with a standard of this Nod factor (kindly provided by Prof. G. Stacey, Center for Legumes Research, University of Tennessee, Knoxville, USA). A second peak with retention time of 32.56 min was also purified by preparative HPLC. Mass spectrometry analysis (electrospray in negative mode) carried out at the Biomedical Mass Spectrometry Unit, McGill University (Dr. O. Mamer) allowed us to identify this peak as Nod Bj-V (Ac, $C_{16:0}$, MeFuc) (Fig. 3.2, chapter three).

4.3.3. Root hair deformation assay

Soybean (cv. OAC Bayfield) seeds were surface sterilized in 2% sodium hypochlorite for 2 minutes and washed thoroughly, several times, with distilled sterilized water. Two seeds were placed on each Petri plate, containing 10 to 20 mL 1.5 % water agar, and incubated in the dark at room temperature. After 5 to 7 days, lateral roots of similar

length and showing abundant root hairs were selected for uniformity of length and aseptically excised and placed on slides containing equivalent concentrations of Nod Bj-V ($C_{18:1}$, MeFuc) and Nod Bj-V (Ac, $C_{16:0}$, MeFuc). The slides were kept in a closed moist chamber for 24 h. Roots were fixed with a staining solution (Prithiviraj et al., 2000). Root hair deformations were observed using light microscopy (Jenalumar, Jena Instruments Ltd, Germany) with observations focused on the root hair susceptible zone (Stokkermans et al., 1995). Each treatment had three replicates. Each experimental unit had three samples of lateral roots, on the same slide. Microscope fields were counted along the entire length of root holding root hairs with focusing on the root zone susceptible to Nod factors and the counts from all fields were added together for determination of percentage of root hairs deformed. Microscopic observations were also made for the control treatment. Roots showing substantial frequencies of overlapping root hairs were not used for data collection as this overlapping tended to cause root hair deformation by itself; root hair deformations caused by Nod factor were considered only in root hairs not entangled by others (Truchet et al., 1985). With the exceptions just noted, all of the root hairs in each microscopic field were counted.

4.3.4. Soybean root hair response to Nod factors treatment under, salinity, low pH, and low incubation temperature stress conditions

Three studies were conducted to evaluate how soybean root hairs respond to exogenous Nod factor applications under three abiotic stress factors: salinity, low pH, and low incubation temperatures. In each experiment, the treatments consisted of factorial combinations of a control where only distilled sterilized H_2O was used, and three Nod

factor concentrations (10^{-6}, 10^{-8}, 10^{-10} M) where the Nod factor consisted of a 1:1 mixture of Bj-V ($C_{18:1}$, MeFuc) and Nod Bj-V (Ac, $C_{16:0}$, MeFuc). The LCO material was freeze dried and dissolved in distilled sterilized water to produce the required LCO solutions. Modifications were applied, based on the type of stress to be tested, as follows:

To test the effect of low incubation temperature on Nod factor bioactivity a study was carried out with three incubation temperatures (15, 17, and 25 °C). The range of incubation temperatures was chosen based on the finding of Zhang and Smith (1994), where a progressive delay of 1-2 days $^{\circ}C^{-1}$ in the early stages of soybean infection process was reported as temperature declined from 25 °C (optimum) to 17.5 °C RZTs. Between 17.5 to 15 °C the delay was much larger, at 7-10 days $^{\circ}C^{-1}$. Solutions of pre-chilled Nod factor (10^{-6}, 10^{-8}, 10^{-10}, and 0.0 M) were prepared as previously descirbed and kept at 4 °C. In the control treatment, distilled sterilized water was applied to the lateral roots.

To test the effect of low pH on Nod factor bioactivity, Nod factor solutions (10^{-6}, 10^{-8}, 10^{-10} M) were adjusted to 2 levels of pH (4 and 5) using HCl. Two control treatments were used in this study, Nod factor solution with a control pH of 6 and, similar to the first study, a distilled sterilized water control.

To test the effect of salinity on Nod factor bioactivity, two concentrations of NaCl (100, and 200 mM) were prepared by dissolving the NaCl in distilled sterilized water and mixing this with Nod factor solutions; the final volume was adjusted according to the assigned concentrations. Two controls were applied to the lateral roots: distilled sterilized water, NaCl-free Nod factor solutions.

In the three studies, soybean lateral roots of similar lengths and showing abundant fluffy root hair growth were aseptically excised and placed on microscope slides containing Nod factor solutions. To ensure complete coverage of root hairs extra Nod factors solution was added to each specimen and incubated in the dark in a moist chamber at 25 °C for salinity and low pH stress factors, while for low temperature stress experiments, slides were incubated at three different temperatures (15, 17, and 25 °C). After 24 h root hairs were stained with methylene blue (0.01%) and then scored for HAD under the microscope (Prithiviraj et al., 2000).

4.3.5. Experimental design and statistical analysis

The studies were conducted twice. Because similar data was observed in both experiments, data were combined after applying Bartlett's test for homogeneity of variance and subjected to statistical analysis. In each experiment, the treatments were the result of factorial combinations of two factors, and the treatments were organized following a randomized split-plot design. The main plot units consisted of the temperature, salinity, or pH levels, while Nod factor concentrations formed the subplots. The percent deformation data for Nod factor treated roots were square root (SQRT) transformed prior to statistical analysis (Steel and Torrie, 1980). The control data was excluded from the statistical analysis because root hair deformations were not detectable in this treatment. The Statistical Analysis System (SAS Institute Inc., 1988) was used for analysis of variance. When a significant treatment effect ($P \leq 0.05$) was observed by ANOVA, a least significant difference (LSD) test was conducted to determine differences among means at $P \leq 0.05$.

4.4. Results

4.4.1. Soybean root hair responses to Nod factor

When Nod factor was applied to soybean root hairs, four types of soybean root hair deformations were observed in this study: wiggling, bulging, curling, and branching (Fig. 3.5. chapter three). None of the typical Nod factor induced HAD were seen in the control treatment slides. In general, soybean root hair deformation responded in a concentration-dependant manner. Nod factor treatment increased root hair deformation, relative to the controls, in all experiments.

4.4.2. Biological activity of Nod factor at low incubation temperature

The frequency of root hair deformation was dose-dependant. In general, 10^{-6} M Nod factor provoked the highest response (28.5%) of root hair deformations over a range of tested Nod factor. As the Nod factor concentration declined, marked reductions in HAD were observed, where 20.6 and 14.5 % was the lowest levels for Nod factor treatments at 10^{-8} and 10^{-10} M (Fig. 4.1). Our data is in agreement with previous studies with respect to the values of root hair deformations evoked by a variety of Nod factor and leguminous plants. There was no interaction between Nod factor and incubation temperature. Sub-optimal incubation temperature markedly affected the response of soybean root hairs to Nod factor. As temperature decreased lower frequencies of root hair deformations were detected, at 17 °C, HAD was 82% of the control (plant roots incubated at 25 °C), while, at 15 °C this was only, 64% (Fig. 4.1).

4.4.3. Biological activity of Nod factor under low pH conditions

In these experiments, again, no interaction between Nod factor and pH effect was detected. Over all, fifty one percent of soybean root hairs were deformed when treated with 10^{-6} M Nod factor at pH 6. Root hair deformation decreased as Nod factor concentration decreased; average of 34 and 19.7% of root hairs were deformed by 10^{-8} and 10^{-10} M Nod factor, respectively (Fig. 4.2). The smallest response to Nod factor solution occurred at pH 4, with 29.8% of root hairs deformed. The degree of response at pH 5 was 37.8% HAD (Fig. 4.2).

4.4.4. Biological activity of Nod factor under salinity stress

There was an interaction between salinity and Nod factor treatment. All Nod factor treatments elicited root hair deformation for the control treatment; the greatest response of soybean root hairs to Nod factor treatment resulted from treatment with salt-free Nod factor solution where the proportional HAD was 42, 27, and 14 % at 10^{-6}, 10^{-8}, 10^{-10} M, respectively (Fig. 4.3). The biological activities of NaCl-amended Nod factor solutions were markedly diminished at the two tested salt concentrations (200 and 100 mM) and there were not differences.

In the absence of salt stress, root hair deformation increased as Nod factor concentration increased. Salinity reduced these responses and there was no difference among Nod factor concentrations under saline conditions. At 10^{-10} M Nod factor the

degree of root hair deformation was not different between the control and Nod factor treatments (Fig. 4.3).

4.5. Discussion

Nod factor, when applied to soybean roots, caused root hair deformations in a dose-response pattern. Because root hair curling induced by Nod factor requires the physical presence of rhizobia for the redirection of root hair tip growth that results in the classic curling (Lhuissier et al., 200), and since Nod factor was applied in the absence of bacteria in these studies, we evaluated all types of root hairs deformation all together and considered this to represent HAD active for Nod factor. A range of deformations were observed. As previously reported for other legumes, (Sanjuan et al., 1992; Relic et al., 1993; Stokkermans et al., 1995; Prithiviraj et al., 2000) where root hair branching was the least common reported deformation. When the biological activity of single Nod factor, purified form *B. elkanii* USDA61 and *B. japonicum* USDA110 and 532C, such as, the main Nod factor Nod Bj-V ($C_{18:1}$, MeFuc) was evaluated on *G. soja* and *G. max* root hairs, no branching response was reported (Sanjuan et al., 1992; Prithiviraj et al., 2000; Duzan et al., chapter three). Nod Bj-V (Ac, $C_{16:0}$, MeFuc) from *B. japonicum* strain 532C triggered the three most common deformations of soybean root hairs (unpublished data), but did not cause branching. It is possible that the intensity and distinctness of the response of soybean root hairs to Nod factor application observed in the current study, and especially branching, requires a specific combination of Nod factor. Partially purified *Rhizobium sp.* NGR234 Nod factor elicited curling and branching of *M. atropurpureum* root hairs (Relic et al., 1993), which might be related to the known ability of strain

NGR234 to produce a wide range of Nod factor, collectively active on many host plants (Price et al., 1992), however, branching was detectable on *Vicia sativa* root hairs, even though a single Nod factor, Nod Rlv-V ($C_{18:4}$, Ac), was applied (Heidstra et al., 1994). Root hair deformations of *Vicia sativa* were also detectable following treatment with a broad range of Nod factor (Carlson et al., 1993).

In this study we tested the biological activity of a 1:1 mixture of two Nod factor form *B. japonicum* 532C. Previous studies showed that the greatest number of nodules were formed when a mixture of two Nod factor (around 60:40) was applied to alfalfa root systems (Truchet et al., 1991). Further, *enod2* expression in soybean roots, an early events involved in nodule initiation, was detected only when a mixture of Nod Bj-V ($C_{18:1}$, MeFuc) and Nod Bj-V ($C_{16:0}$, MeFuc) were used, and their combined effectiveness was ratio dependant, requiring an equal or higher proportion of Nod Bj-V ($C_{18:1}$, MeFuc), the main Nod factor produced by microsymbionts of soybean (Minami et al., 1996).

The impact of suboptimal root zone temperatures on soybean-bradyrhizobia associations, particularly at early stages of symbiosis, has been addressed in a number of studies (Zhang and Smith, 1994, 1995, 1996; Zhang et al., 1996); here we present evidence of negative effects of low temperature on the bioactivity of Nod factor. Previously, we addressed the negative effect of low growth temperature on Nod factor production by *B. japonicum* 532C and USDA110, a prior step in the symbiotic recognition process (Duzan et al., chapter three). Our data corroborates the low temperature inhibitory effect on the early stages of nodulation, specifically, on root hair

curling, the first visible step in nodulation, and the step immediately following Nod signal excretion by rhizobia in the presence of a compatible legume partner.

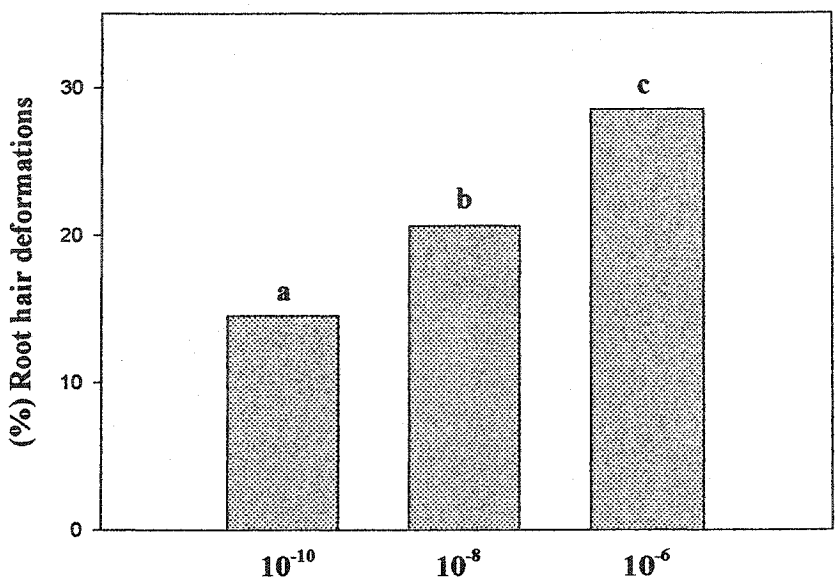
There was a reduction in Nod factor activity at under low pH conditions. This is in agreement with previous observations regarding the low pH-sensitivity of early steps in the nodulation process including, root hair deformation, *nod* gene expression, Nod factor production, and root hair curling, all of which are probably related to effects on both rhizobia and the plant root system (Evans et al., 1980; Richardson et al., 1988a; McKay and Djordjevic, 1993; Staley, 2003). In white clover, morphological parameters of root growth and development, such as, total length, diameter, tip number, and surface area were not affected by low soil pH, while, rhizobia proliferation and function were, suggesting that the inhibitory effect of low pH occurs at the microbial level, affecting nodule primordia initiation (Staley, 2002; Staley, 2003). In this study, only the external response of soybean root hairs to Nod factors application under low pH stress was tested.

Salt stress decreased the soybean root hair responses to Nod factor. This is reasonable as salinity can directly affecting the legume restrict root growth, affecting the responses of roots to rhizobia and Nod factor (Tu, 1981). Salinity levels of 255 and 340 mM NaCl caused shrinkage of soybean root hairs, affecting the early stages of symbiosis, particularly root hair deformation (curling), resulting in inhibition of infection process, respectively (Tu, 1981; Elsheikh, 1998). There may be other, less direct, environmental effects on nodulation due to low pH, such as limiting the amount of Nod factor excreted by the microsymbiont through reduced availability of phosphate (McKay and Djordjevic,

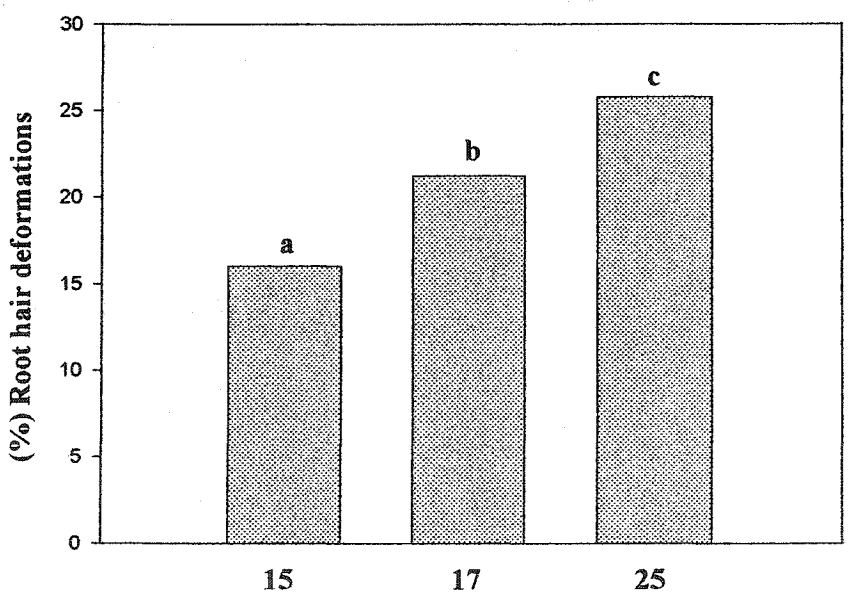
1993). Minor effects of osmolarity have been reported on *nod*D and *nod*ABC expression in *B. japonicum* (Wang and Stacey, 1990).

In conclusion, to our knowledge, this is the first study addressing the effect of abiotic stresses on Nod factor perception by root hairs. Under control conditions, and in the presence of low pH and low temperature stresses, the degree of root hair deformation increased with increasing Nod factor concentration, and diminished as the applied stress increased. For low RZT and pH stresses there was no interaction between Nod factor level and stress level. However, for salinity the increase in root hair deformation with increasing Nod factor concentration occurred in the control, but not when salt stress was present. Thus, adding additional Nod factor helped overcome the measured stress effect associated with low temperature and low pH, but this is not the case for salinity.

Fig. 4.1. (A) Root hair deformations as a function of Nod factor concentrations. (B) Root hair deformations at three incubation temperatures. Bars followed by a different letter are significantly different, by an ANOVA ($p = 0.05$) protected $LSD_{0.05}$ test. Note: There was no interaction between Nod factor and temperature).

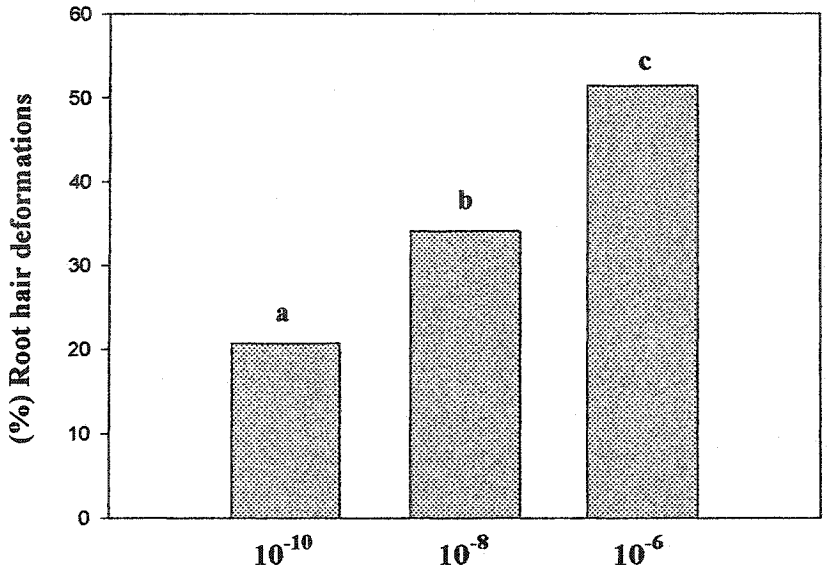


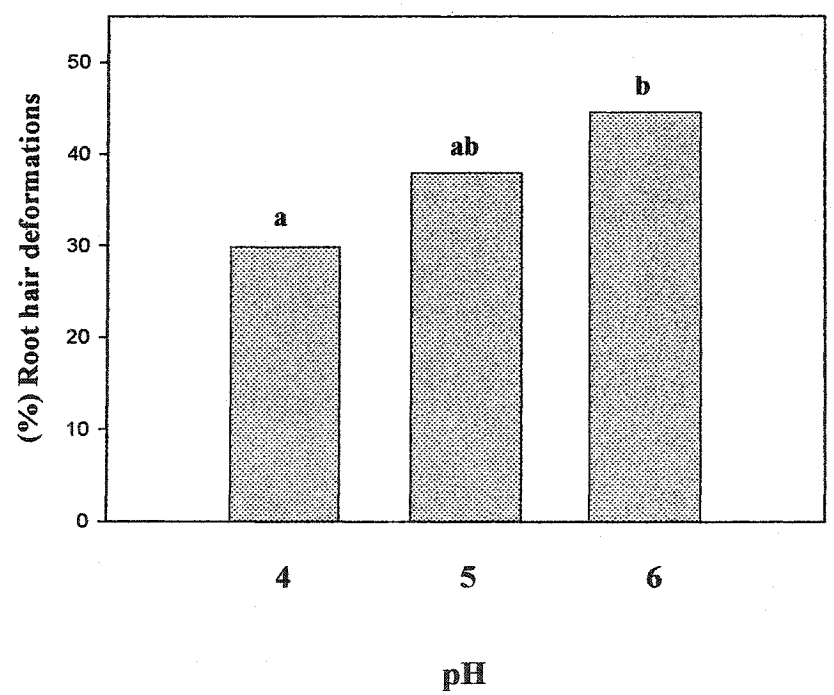
Nod factor concentrations (M)



Temperature (°C)

Fig. 4.2. (A) Root hair deformations as a function of Nod factor concentrations. (B) Root hair deformations tested at different pH levels. Bars followed by the same letter are not significantly different, by an ANOVA ($p = 0.05$) protected $LSD_{0.05}$ test. Note: There was no interaction between Nod factor and pH.


60
50
40
30
20
10
0
(%) Root hair deformations
a
b
c
10-10
10-8
10-6
Nod factor concentrations (M)


50
40
30
20
10
0
(%) Root hair deformations
a
ab
b
4
5
6
pH

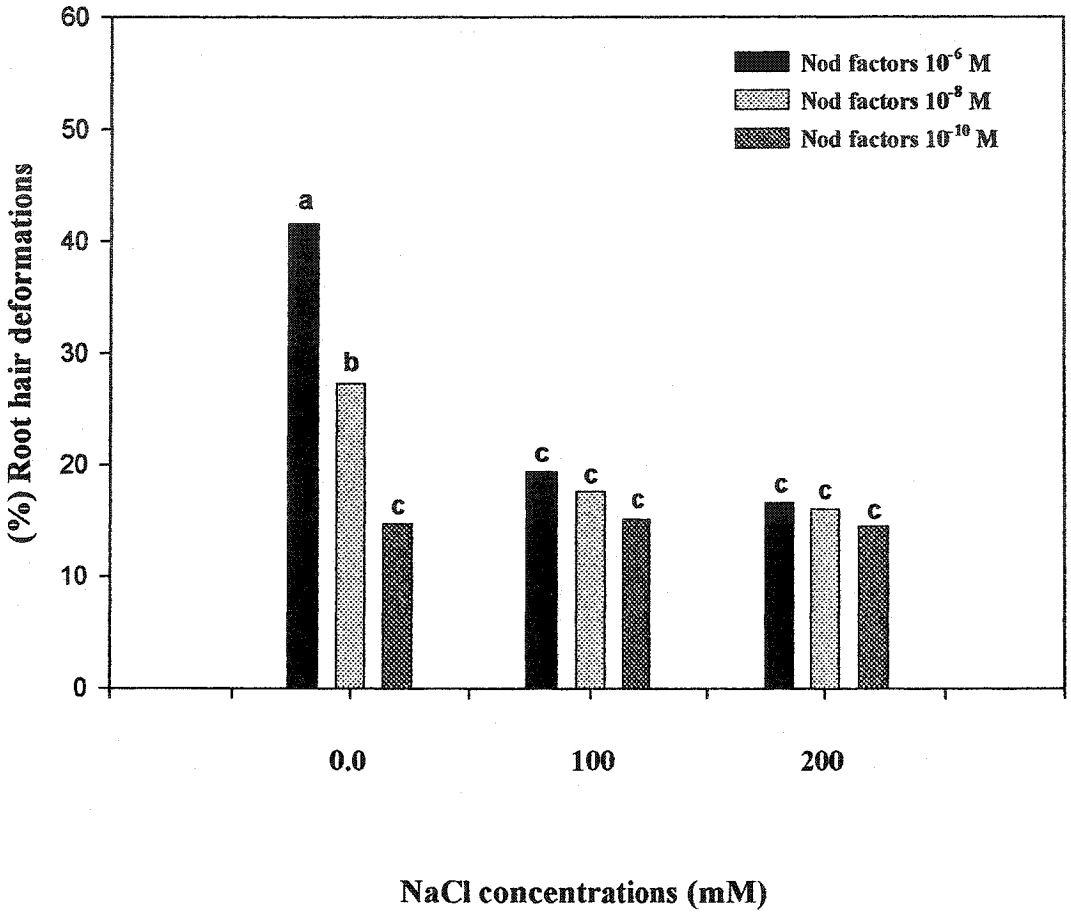


Fig. 4.3. The effect of various salt concentrations on Nod factor bioactivity. Bars followed by the same letter are not significantly different, by an ANOVA (p = 0.05) protected $LSD_{0.05}$ test.

Preface to Chapter V

Chapter 5 is compromised of a manuscript prepared by H. Duzan, Dr. X. Zhou, Dr. A. Souleimanov, and Dr. D.L. Smith, to be submitted to Molecular Plant-Microbe Interactions for publication. The format has been changed to be consistent within this thesis. All literature cited in this chapter are listed in the reference section at the end of the thesis. All Figures for this chapter are presented at the end of this chapter.

The contributions of the co-authors are described in details in the section of contributions of authors.

In the previous two chapters we examined the effects of abiotic stresses on the ability of *B. japonicum* cells to produce Nod factor, and soybean roots to recognize and respond to Nod factor, and found that abiotic stresses inhibit both these elements of signal between soybean and *B. japonicum*. In this chapter we investigated the ability of *B. japonicum* Nod factor to reduce the effects of a biotic stress (powdery mildew) on soybean, and its potential role in inducing resistance in soybean against the plant pathogen causing powdery mildew, and the effects of low RZT on this response. Also, the effect of Nod factor application on the activities of phenylalanine ammonia lyase (PAL specific activity)

Chapter V

Application of Nod Factor Induces Resistance in Soybean Against Powdery Mildew

H. M. Duzan, X. Zhou, A. Souleimanov and D.L. Smith*

*Department of Plant Science, Macdonald Campus of McGill University, 21, 111 Lakeshore, Sainte-Anne-de-Bellevue, QC, H9X 3V9, Canada

5.1. Abstract

Plants possess highly sensitive perception systems by which signal molecules can be recognized, which in turn induce various plant responses. In the *Bradyrhizobium*-soybean (*Glycine max* (L.) Merr.) symbiosis, the recognition process is initiated through mutual exchange of signal molecules, generally flavonoids from soybean plants and lipo-chitooligosaccharides (Nod factor) from the microsymbiont. Application of the Nod factor Nod Bj-V ($C_{18:1}$, MeFuc) induced soybean resistance against powdery mildew caused by *Microsphaera difussa* at two RZTs (17 and 25 °C). Addition of Nod factor, at concentrations ranging from 10^{-6} to 10^{-10} M, to soybean root systems led to reductions in disease incidence at both RZTs. The lowest disease incidence was caused by Nod factor treatment at 10^{-6} M and this effect persisted throughout the experiment for plants kept under a 17 °C RZT. The effect of Nod factor application on fungal growth and development was measured at 4, 12, 48, and 96 h after inoculation. Colony diameter and number of germ tubes per conidium were decreased by 10^{-6} M Nod factor treatment, especially for plants kept at 17 °C RZT. Treatment of soybean seedlings with Nod factor, through stem wounds, induced phenylalanine ammonia lyase (PAL) activity. The most rapid increase in PAL followed treatment with 10^{-6} M Nod factor, the highest concentration tested.

5.2. Introduction

World population and food demand continue to rise, resulting in a need for increased crop production (Wennemann, 2002). Over the last 50 years, global crop production has kept up with world population, however, as crop protection has intensified the use of potentially environmentally damaging inputs, such as fungicides, insecticides and herbicides, has increased; there is a need to develop crop production methods that produce high yields but, at the same time, require fewer inputs, making them more sustainable in the long term.

Soybean (*Glycine max* [L.] Merr.) growth and development can be affected by diverse types of pathogens. In 1998, the total estimated yield loss due to soybean diseases was valued at U.S. $6.29 billion (Wrather et al., 2001), including fungal diseases such as powdery mildew, caused by the obligate foliar parasite *Microsphaera diffusa* Cke. & Pk (Mignucci et al., 1979). Powdery mildew can disrupt leaf structure, leading to declines in photosynthetic activity as great as 50%, and yield losses of up to 35% in naturally infected fields of susceptible soybean cultivars (Dunleavy, 1979; Mignucci and Boyer, 1979; Phillips, 1984). The earliest reported occurrence of soybean powdery mildew is from the U.S. (Lehman, 1931). Recently, serious outbreaks of soybean powdery mildew were reported in eastern Asia (Takamatsu et al., 2002).

The earliest event in the establishment of the rhizobia-legume association is a sophisticated exchange of signal compounds, required for a successful symbiosis. Plant roots exude signal molecules, mainly flavonoids, which activates the Nod D protein. This

protein causes the induction of bacterial *nod* gene expression resulting in the synthesis of bacteria-to-plant signal molecules, Nod factor, which play a vital role in the early events of the legume-rhizobia symbiosis (Lhuissier et al., 2001). The exact structure of the Nod factor produced varies among rhizobial strains, and this is a component of specificity in the rhizobia-legume nitrogen fixing symbiosis (Schultze and Kondorosi, 1998). In general, Nod factor are composed of a tri- to penta-chitin backbone possessing an *N*-acyl group at the non-reducing end and a variety of substitutions along the chitin backbone. The structure of the acyl chain and substitutions on the chitin backbone play a key role in specifying compatibility of rhizobial strains with macrosymbiont species, allowing recognition by the correct plant host (Schultze and Kondorosi, 1998). The main Nod factor produced by the soybean microsymbiont, *Bradyrhizobium japonicum* is Nod Bj-V ($C_{18:1}$, MeFuc) (Sanjuan et al., 1992; Souleimanov et al., 2002).

Application of appropriate Nod factor to soybean roots evokes a number of physiological responses, such as plasma membrane depolarization (Ehrhardt et al., 1992), calcium spiking (Felle et al., 2000), root hair deformations (Heidstra et al., 1994; Prithiviraj et al., 2000; Duzan et al., chapters three and four), induction of incipient nodule meristems (Stokkermans and Peters, 1994), and enhancement of early growth stages of legume and non-legume plants (Souleimanov et al., 2002). When extremely low concentrations of purified Nod factor were applied to *G. soja* roots, complete, although empty of rhizobia, nodule structures developed (Stokkermans and Peters, 1994).

It is interesting to note that the signaling occurring at the beginning of the N_2 fixation symbiosis involves the exchange of flavonoids and chitin based compounds. Plants have a well characterized ability to detect chitin fragments, elicitors of plant

defense reaction and constituents of fungal cell walls, and to produce phytoalexins in response (Ebel and Mithofer, 1998). Thus, it may be that during the course of evolution a hostile signaling system, established to detect and respond to the presence of pathogenic fungi, has been converted to a friendly signaling system, used in the establishment of an extremely important symbiosis. It may also be that vestiges of the original protective function remain in the symbiotic signaling system.

In addition to the reported effects of rhizobia on plants, the potential role of rhizobia in the biological protection of the macrosymbiont against pathogenic organisms was recently reviewed by (Dakora, 2003). Legumes grown in soil treated with rhizobia were less affected by a number of fungal pathogens, such as, *Phytophthora megasperma, Fusarium solani* f.sp. phaseoli (Tu, 1978; Buonassissi et al., 1986). *In vitro*, reduced sporulation of *Phytophthora megasperma, Pythium ultimum, Ascochyta imperfecta*, and *Fusarium oxysporum* was reported when *B. japonicum* was inoculated onto the culture medium (Tu, 1979). Under field conditions, soil inoculation with rhizobial strains resulted in protection of some crops, including soybean, against *Rhizoctonia solani*, *Macrophomina phaseolina*, and *Fusraium spp.* (Ehteshamul-Haque and Ghaffar, 1993). Though it is not clear how rhizobia act against pathogens Tu (1979) suggested that they directly interfere with the early stages of the pathogenic infection, inhibiting the attachment of the pathogen to the host plant, perhaps by parasitizing the hyphal tips of the fungus.

Following recent advances in understanding of the signaling processes involved in establishment of the symbiotic association between rhizobia and leguminous plants,

increasing evidence suggests roles for rhizobial Nod factor that go beyond the nodulation process. Nod factor are known to affect phytoalexin concentrations in plant tissues (Dakora, 2003). Plant isoflavonoid biosynthesis was stimulated by the presence of rhizobial cells (Dakora et al., 1993). Further, an increase in medicarpin, a phytoalexin of *Medicago sativa,* was observed following cell culture treatment with 10^{-6} M Nod Rm-IV ($C_{16:2}$, S) but not desulfated Nod Rm-IV ($C_{16:2}$). Nod factor also induced expression of genes encoding enzymes of the isoflavonoid biosynthetic pathway in *Medicago* roots, including chalcone synthase, chalcone reductase, isoflavone reductase and a pathogen related protein (Savoure et al., 1997).

A key enzyme in the phenylpropanoid pathway is phenylalanine ammonia-lyase (PAL), which initiates the first reaction in the pathway, resulting in the formation of cinnamic acid (Dixon et al., 1995); cinnamic acid is metabolized to supply intermediates for the synthesis of secondary metabolites such as phytoalexins. Phytoalexins are characterized by antimicrobial activities (Morrison and Buxton, 1993). PAL activity can be induced during plant-pathogen interactions as well as by non-pathogenic agents, such as environmental conditions (Dixon and Paiva, 1995). Infection of soybean roots with *B. japonicum* induces PAL genes and this has been attributed to post infection events (Estabrook et al., 1991). PAL activity can also be stimulated by a number of elicitors, including chitin and chitosan. Recently, the stimulation of PAL activity in soybean by chitin and chitosan oligomers was observed in soybean, suggesting the induction of this defense-related metabolic pathway in soybean plants (Khan et al., 2003). In the present study we investigated the capacity of Nod Bj-V ($C_{18:1}$, MeFuc) to induce PAL activity and powdery mildew resistance in soybean plants.

5.3. Materials and Methods

5.3.1. Plant materials

Plants were produced following, in general, the methods of Zhang and Smith (1994). Briefly, soybean cv. OAC Bayfield seeds were surface sterilized in 2% sodium hypochlorite for ~ 2 minutes and washed several times with distilled sterilized water. The seeds were planted in trays containing sterilized vermiculite. After about four days, 3 germinated seeds showing vigorous seedling growth were selected and transferred together into a growth pouch; the pouches were then placed in one of 4 tanks with two tanks set at 17 $\pm$ 2 °C root zone temperature (RZT), and two at 25 $\pm$ 2 °C RZT. RZTs were controlled by circulating cooled water around the growth pouches. After four days two of the three seeds in each pouch were removed; the ones left were selected for uniformity of growth.

5.3.2. Bacterial culture

Bradyrhizobium japonicum 532C was obtained from Liphatech Inc. (Milwaukee, WI, USA). This strain was selected as it has been widely used in commercial *B. japonicum* inoculants in Canada. The culture was grown at 28 °C in 250 mL yeast extract mannitol broth (YEM), with shaking at 150 rpm, for 4 to 6 days, and thereafter for production of Nod factor on a larger scale bacteria were subcultured into 2 L (25 mL per 2000 mL of YEM medium). After 7 days of subculture (OD_{620} 0.4-0.6), Nod factor synthesis and production by bacterial cultures was induced through the addition of 5 µM genistein and

further, bacterial cultures were incubated for an additional 48 to 96 h, followed by Nod factor extraction. In addition, a non-induced bacterial culture was prepared, to act as a control for HPLC analysis. This was subject to the same Nod factor isolation procedure as the induced cultures.

5.3.3. Nod factor extraction and purification

The 2 L culture was extracted with 40% HPLC-grade 1-butanol by shaking the mixture for 5 to10 minutes and then allowing the two phases to separate for 24 h. The organic phase (butanol layer) was collected and evaporated at 80 °C in a Yamato RE500 Rotary evaporator (Yamato Scientific American Inc., NY, USA). The resulting material (volume of 2 to 3 mL) was dissolved in 4 mL of 18% of acetonitrile and stored in the dark in glass tubes at 4 °C for 24 h. Samples were then centrifuged for 10 minutes at 12,000 g, and the supernatant was collected for HPLC analysis.

For analytical purposes, during the first run, two hundred μL of the Nod factor extract were injected into a Waters HPLC system (Waters Associates Inc., Milford, MA) consisting of a model 712 WISP HPLC, fitted with two model 510 pumps and a model 441 UV detector operated at 214 nm. Separation was carried out with a Vydac C18 reversed-phase column (5 μm, 46 X 250 mm, Vydac, USA). To elute Nod factor from the column, a program of acetonitrile and water gradients was used: 18% acetonitrile (10 min), a linear gradient from 18 to 60% acetonitrile (20 min), a linear gradient from 60 to 100% acetonitrile (5 min). A chromatographic peak was identified as Nod Bj-V ($C_{18:1}$, MeFuc) by comparing its retention time with a standard of this Nod factor (a gift from

Prof. G. Stacey, Center for Legumes Research, University of Tennessee, Knoxville, USA). The identity of this peak has also been confirmed by mass spectrometry. For Nod factor production on a large scale, the previous method was applied except that two mL was injected into the HPLC system. The resulting Nod factor was freeze-dried and redissolved in distilled sterilized water.

5.3.4. Effect of temperature and Nod factor concentration on disease intensity

In this experiment, four levels of Nod Bj-V ($C_{18:1}$, MeFuc) were tested. Seedlings were maintained in the growth pouches and watered with sterilized H_2O for the first week, after which plants were watered with nitrogen-free Hoaglands solution (Zhang and Smith, 1995). Because low temperature was one of the stress factors tested in relation to Nod factor production and activity in the other chapters of this thesis, and because low RZT is known to inhibit stages of the infection process during establishment of the soybean-bradyrhizobia symbiosis (Zhang and Smith, 1994), two RZTs [17 °C (critical temperature for soybean nodulation and nitrogen fixation) and 25 °C [optimum temperature for soybean nodulation and N_2 fixation (Zhang et al., 1995)] were included in this experiment. The experiment was organized following a completely randomized split-plot design. Temperature was the main plot factor. The subplot factor was Nod factor concentration (10^{-6}, 10^{-8}, 10^{-10}, 0.0 M) with 2 replicates per treatment. Photoperiod of 16/8 h day/night cycle at 25 ± 2 °C, and ~ 75% relative humidity was maintained throughout the experiment. Nod factor was added to each growth pouch with a sterile syringe. Non-induced *B. japonicum* was cultured and maintained on YEM (Vincent, 1970). OD was adjusted to an A_{620} of 0.08 (approximately 10^8 cells mL^{-1}) with sterilized

water, adjusted to temperatures appropriate to the treatment RZTs, and 1 mL was applied, with a sterilized syringe, onto each plant root system. Inoculum from naturally infected soybean was maintained by infecting soybean leaves that were grown in a separate room, under the same conditions, in the greenhouse. Given the type of symptoms caused by powdery mildew infection and the presence of sign of symptoms, the anamorph stage of the fungus on soybean leaves which were confirmed under the microscope. There was no other fungal growth or secondary infection on soybean leaves. Because Nod factor stimulate transcription of genes encoding enzymes of plant defense-related responses over at least a 48 h period (Savoure et al., 1997) powdery mildew fungus was applied to soybean leaves 48 h after Nod factor addition to soybean root systems. Soybean leaves heavily infested with powdery mildew were used to inoculate the test plants when the first trifoliate leaf was fully developed (Lohnes and Nickell, 1994) by tapping the infected leaves over the leaves of the test plants, thus depositing spores on the surface of the leaves of the test plants. The inoculated plants were monitored for initial signs of powdery mildew and once initial disease symptoms were evident, data on disease incidence were taken weekly for three weeks. By three weeks after inoculation powdery mildew infection reached 100% on at least some plants in most treatments at 25 °C RZT. The degree of infection (% of disease intensity) was scored from 0 to 5 according to percent of leaf tissue showing powdery mildew symptoms, with 0 = none, 1 = trace to 10%, 2 = 10.1 to 25%, 3 = 25.1 to 50%, 4 = 50.1 to 75%, 5 = over 75% (Singh and Prithiviraj, 1997). Disease intensity was calculated for each replicate per treatment using the following equation:

Disease intensity = Sum of rating (0 to 5)/ (Maximum possible score x Total number of leaves examined) x 100.

The entire experiment was conducted twice, with similar results in both instances. Data was combined after subjection to Bartlett's test followed by statistical analysis.

5.3.5. Assessment of effect Nod factor on phases of pathogen development

In this experiment, the effect of four concentrations (10^{-6}, 10^{-8}, 10^{-10}, 0.0 M) of Nod factor treatment on fungal growth and development was studied, for control treatment (0.0 M), plants were treated with distilled sterilized water in addition to the bacterial inoculnum *B. japonicum*. As in experiment one, after Nod factor treatment and fungal inoculation, three plants from each treatment, for three replicates, were collected at each sampling, at the two RZTs (17, and 25 °C). Samples were collected from one tank of each RZT. Therefore, there were two experiments, one at each RZT, and the structure of each experiment followed a completely randomized design. The data was collected from the third nodal leaves of each plant at intervals of 4, 12, 48, and 96 h after fungal inoculation. Data were taken on germination at 4 h, germination and appressorium formation at 12 h, number of hyphal tubes per conidium and colony size, measured as the average of the greatest colony diameter, at 48 h, and colony size at 96 h after fungal inoculation. At least, 100 conidia per replicate were observed in order to calculate percent frequency of conidial germination and appressoria formation at 4 and 12 h after fungal inoculation. Collected samples were fixed following the method of Carver and Adaigbe (1990). Briefly, samples were placed carefully into Petri plates containing ethanol/acetic acid 3:1 until completely cleared of chlorophyll, then placed on other filter paper in a Petri plate containing lactoglycerol (glycerol: lactic acid: water; 1:1:1; v:v). Slides were prepared with methylene blue (0.01%) for microscopic observations. Data were subjected to

angular transformation (Frey and Carver, 1998) before statistical analysis. The experiment was conducted twice with similar results at the both instances.

5.3.6. Enzyme assay (PAL)

5.3.7. Plant materials

Assays from PAL activity followed the method of Khan et al. (2003), for both treatment of plant materials and application of treatments. Briefly, soybean cv. OAC Bayfield seeds were surface sterilized using 2% sodium hypochlorite for approximately 2 minutes, rinsed thoroughly with distilled sterilized water, and then planted in sterilized perlite in a greenhouse under a 16/8 h day/night cycle at 25 ± 2° C, and ~ 75% relative humidity.

5.3.8. Nod factor treatment

Plants at the first trifoliate stage were selected for growth uniformity. Plant stems were excised using scalpel, cutting each at the base of the stem and placing each into 4 mL glass test tubes containing 0.5 mL of Nod factor solution. When the solution was completely taken up, seedlings were immediately transferred into glass tubes containing distilled water. Plants were kept under constant light (85 $\mu mol.m^{-2}\ s^{-1}$). The leaves of three plants, representing each of the three blocks of each treatment, were collected at regular intervals (4, 12, 24, 48, and 96 h) after Nod factor treatment, weighed, and stored immediately at –80 °C.

5.3.9. Enzyme extraction

Leaf samples (300 mg fresh weight) were ground with a cold mortar and pestle containing 4 mL of buffer (50 mM Tris pH 8.5, 14.4 mM 2-mercaptoethanol, 5% w/v insoluble polyvinylpolypyrorolidone). Samples were kept on ice at all times. Extracts were centrifuged at 6,000 g for 10 minutes at 4 °C and the supernatant was collected and centrifuged at 10,000 g for an additional 10 minutes at 4 °C.

5.3.10. PAL enzyme assay

PAL activity was assayed as described by Beaudoin-Eagan and Thorpe (1985); 100 µL of enzyme preparation was mixed with 500 µmol of Tris-HCl buffer (pH 8.5), and 6 µmol of *L*-phenylalanine in a final volume of 1 mL of reaction mixture and incubated at 40 °C for 60 min. A blank with no *L*-phenylalanine was also prepared. The reaction was stopped by the addition 50 µL of 5 N HCl. The product (trans-cinnamic acid) was detected and quantified by measuring absorbance at 290 nm against a blank. Enzyme activity was expressed in nM (trans-cinnamic acid) mg protein^{-1} min^{-1}, where 1 U is defined as 1 nM (trans-cinnamic acid) mg protein^{-1} min^{-1}. The total protein concentration in enzyme extracts was determined by the Bradford method (Bradford, 1976).

5. 3. 11. Statistical analysis

Data on disease intensity and phases of fungal growth and development were analyzed through analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute Inc., 1988). Treatment effects were considered significant when detected at $P \leq 0.05$. When this occurred a least significant difference (LSD) test was conducted to test for differences among means ($P < 0.05$).

5.4. Results

5.4.1. Effects of Nod factor on disease intensity

Nod factor treatment slowed disease development at all as indicated by disease intensity at the first sampling (at one week after inoculation). At 17 °C RZT, a reduction in disease intensity was detected for all Nod factor concentrations, with the least development of disease symptoms occurring at the highest Nod factor concentration (10^{-6} M, Fig. 5.1). Disease intensity in the control treatment was approximately three fold higher than for the 10^{-6} M Nod factor treatment. A similar pattern was observed at RZT 25 °C; at higher Nod factor concentrations the incidence of disease was 75 % lower for the 10^{-6} M Nod factor treatment than the control (Fig. 5.1. a).

The disease intensity at two weeks after inoculation increased for all treatments, however, the 10^{-6} M Nod factor treatment continued to have the lowest level of powdery mildew infection at both RZTs (Fig.5.1. b).

By the third observation (third week) disease intensity had increased markedly. The overall progression of disease was slower for the lower than the higher RZT and at the higher RZT infection levels were high and not different among Nod factor concentrations. At 17 °C RZT plants treated with 10^{-6} M Nod factor had a lower intensity of disease than the control plants (Fig.5.1. c).

5.4.2. Effect of RZTs on disease development

Disease incidence was always higher at 25 °C RZT than at 17 °C RZT. There was an interaction between Nod factor and RZT at the first sampling (1 week after inoculation). At the second and the third samplings there were no differences among treatments at 25 °C RZT, but differences persisted at 17 °C RZT (Fig. 5.1. a).

5.4.3. Effect of Nod factor on fungal growth at 17 °C RZT.

There was no evidence that Nod factor treatment affected conidial germination or appressorium formation by *M. diffusa* on soybean leaves at 4 and 12 h after inoculation. However, a reduction was observed in colony diameter and number of hyphal tubes per conidium due to treatment with 10^{-6} M Nod factor 48 h after inoculation. At 96 h after inoculation, colony diameter was markedly reduced by all Nod factor treatments, as compared to the control treatment, and this effect was dose-dependant, the greatest reduction in colony size occurring at 10^{-6} M, the highest Nod factor concentration tested (table 5.1).

5.4.4. Effect of Nod factor on fungal growth at 25 °C RZT.

Nod factor did not affect fungal conidial germination on soybean leaves 4 and 12 h after inoculation, while appressorium formation was reduced to some extent 12 h after inoculation. By 48 h after inoculation reduced colony size and number of germ tubes per colony were observed at the highest Nod factor concentration (10^{-6} M), compared to the control (table 5.2). By 96 h after inoculation an over lapping of fungal colonies was observed and, therefore, it was not possible to take data of the colony size.

5.4.5. PAL response to Nod factor treatment

PAL specific activity was induced in soybean leaves by all Nod factor treatments of excised leaves. Although, biphasic and differential amplitudes and timing of PAL activity induction was measured among tested levels of Nod factor. At 24 h leaves collected from plants treated with 10^{-6} M Nod factor exhibited a maximum level of PAL specific activity (23.2 U) followed by 10^{-8} M (19.2 U) and 10^{-10} M (18.9 U), all of which were greater than the control plants (16.9 U). After 48 h PAL activity declined for the 10^{-6} M treatment (20.6 U), although it remained higher than the control, while levels continued to increase for 10^{-8}, 10^{-10} M treatments (20.2 U, and 19.2 U, respectively), although the latter was not greater than the control (18.4 U) at this time. At 96 h plants treated with 10^{-10} M showed the highest PAL specific activity (26 U) and exceeded the control (Fig. 5.2).

5.5. Discussion

In this study the role of Nod factor in inducing plant-defense reactions was studied. Our data are at least partially in agreement with the findings of (Savoure et al., 1997) where Nod factor-mediated defense reaction was observed in *Medicago* cell cultures and roots. Felle et al. (2000) reported data not completely in agreement with Savoure et al. (1997). Felle et al. (2000), reported that an increased concentration of cytosolic calcium caused by Nod factor treatment of alfalfa root hairs did not exceed the (hypothetical) threshold required for signal transduction associated with activation of defense-related reactions, when compared to the effect of chitin oligomer. The difference between the findings of Savoure et al. (1997) and those of Felle et al. (2000) was attributed to the lower concentration of Nod factor applied to alfalfa root hairs by Felle et al. (2000) (10^{-7} M), as compared to the higher Nod factor concentration (10^{-6} M) applied to *Medicago* cell cultures in the work of Savoure et al. (1997). On the other hand, alkalinization of soybean suspension cultures, caused by Nod Bj-V ($C_{18:1}$, MeFuc), the same Nod factor tested in the current study, was felt to suggest elicitation of a defense-related response (Day et al., 2001).

In the present study, the lowest incidence of disease occurred when plants were treated with 10^{-6} M Nod factor. This concentration has been shown to evoke defense related responses in *M. sativa* roots in the form of induced expression of a set of genes related to isoflavonoid biosynthesis and pathogen related proteins (Savoure et al., 1997), and non-pathogenic responses in the form of root hair deformations (Prithiviraj et al., 2000; Duzan et al., chapter four).

It has been shown that Nod factor treatment suppresses endogenous salicylic acid levels in soybean plants (Martinez-Abarca et al., 1998), which suggests that host defense responses might be compromised during the early stages of symbiosis. This may explain how rhizobia are able to successfully infect legume roots, in that they may inhibit the SA-dependent host plant defense responses. However, this suppression of SA related defense responses might be associated with concomitant activation of other defense responses that protect the soybean plants during these early stages of their growth. In some cases, up regulation of jasmonate disease resistance signaling results in down regulation of SA signaling (Dong, 1998). Methyl jasmonate-mediated induction of powdery mildew resistance has recently been demonstrated in barley leaves, and this was associated with marked increases in PAL activity (Walters et al., 2002). Thus, Nod factor treatments may be activating one set of disease responses and inhibiting another.

There was an effect of Nod factor treatment on later stages of fungal growth, such as colony size and secondary growth parameters, particularly, number of hyphal tubes per conidium, while germination and appressorium formation were not affected at either RZT. Primary stages of powdery mildew growth, such as germination and appressorium formation, in general, are not affected by elicitor treatments (Singh and Prithiviraj, 1997; Frey and Carver, 1998). Germination and appressorium formation do not necessarily indicate successful infection, however, establishment of biotrophy by powdery mildew can be observed by 4 h after inoculation, indicating the formation of functional haustoria, which support further secondary fungal growth (Smith et al., 1996). At 17 °C RZT and 96 h after inoculation, colony diameter was smaller for all Nod factor treatments than the control, indicating indirect effects on haustorium function. Haustoria are the interface

between fungus and plant host, through which parasitism is supported by unidirectional flow of nutrients to the fungus. At 25 °C RZT, appressorium formation, colony size, and number of haphal tubes per conidium were affected at 12 and 48 h after Nod factor treatment. This may have affected infection establishment at later stages. At 25 °C RZT it was not possible to observe the rate of fungal growth, and therefore treatment effects on this variable, by 96 h after inoculation.

PAL is a key enzyme in the phenylpropanoid pathway, catalyzing the first reaction of the pathway and providing precursors for the synthesis of compounds with antimicrobial activity, such as phytoalexins. Application of Nod factor to cut soybean seedling stems increased PAL activity, with the most rapid increase occurring at the highest concentration of Nod factor (10^{-6} M). The lowest level of Nod factor applied (10^{-10} M) caused the slowest increase in PAL activity, but eventually caused the greatest increase. It would appear that the relatively rapid increase in rate of disease incidence, observed with the 10^{-10} M treatment, is not related to induced PAL levels, but may be associated with timing of induction. It may be that the levels of PAL activity at the lowest Nod factor concentration simply rose too slowly and the disease became established before the plant defenses were in place. However, one must be cautious about making direct comparisons between cut tissue and root applications of Nod factor. It has been reported that treatment of cell cultures with 10^{-6} M Nod factor increases production of medicarpin (a phytoalexin with antimicrobial activities) by *M. sativa* (Savoure et al., 1997). Accumulation of flavonoids (genistein, coumestrol, and daidzein) was reported in soybean root exudates treated with 10 nM of Nod Bj-V ($C_{18:1}$, MeFuc, OH) and various types of NodNGR over a range of [10^{-8} – 10^{-10} M] (Schmidt et al., 1994), in keeping with

our findings; flavonoids and medicarpin is synthesized via the phenylpropanoid pathway, through steps downstream from PAL.

In the present study, we have shown that Nod factor induce soybean resistance to powdery mildew, and that this effect depends on Nod factor concentration, with greater concentrations inducing more resistance, at least over the range tested. Because of the chemical similarity between Nod factor and chitin oligosaccharides, known elicitors of plant defense reactions, it is possible that Nod factor induction of plant defense responses is related to Nod factor structure (Baier et al., 1999), particularly the chitin backbone (Day et al., 2001). Foliar spraying and root treatment with chitin oligosaccharide induced resistance of wheat to powdery mildew (YaJun et al., 2001). Moreover, Nod factor and chitooligosaccharide applications to *Medicago* cell suspension cultures elicited transcription of genes encoding enzymes of the phenylpropanoid pathway, which is involved in plant defense reactions (Savoure et al., 1997). On the other hand, Minami et al. (1996) reported that, like Nod factor, chitin oligomer induced the expression of the early nodulin ENOD40 in soybean. In addition, it is known that Nod factor imitate chitin oligosaccharides in their induction of chitinase activity in host and non-host plants (Staehelin et al., 1994).

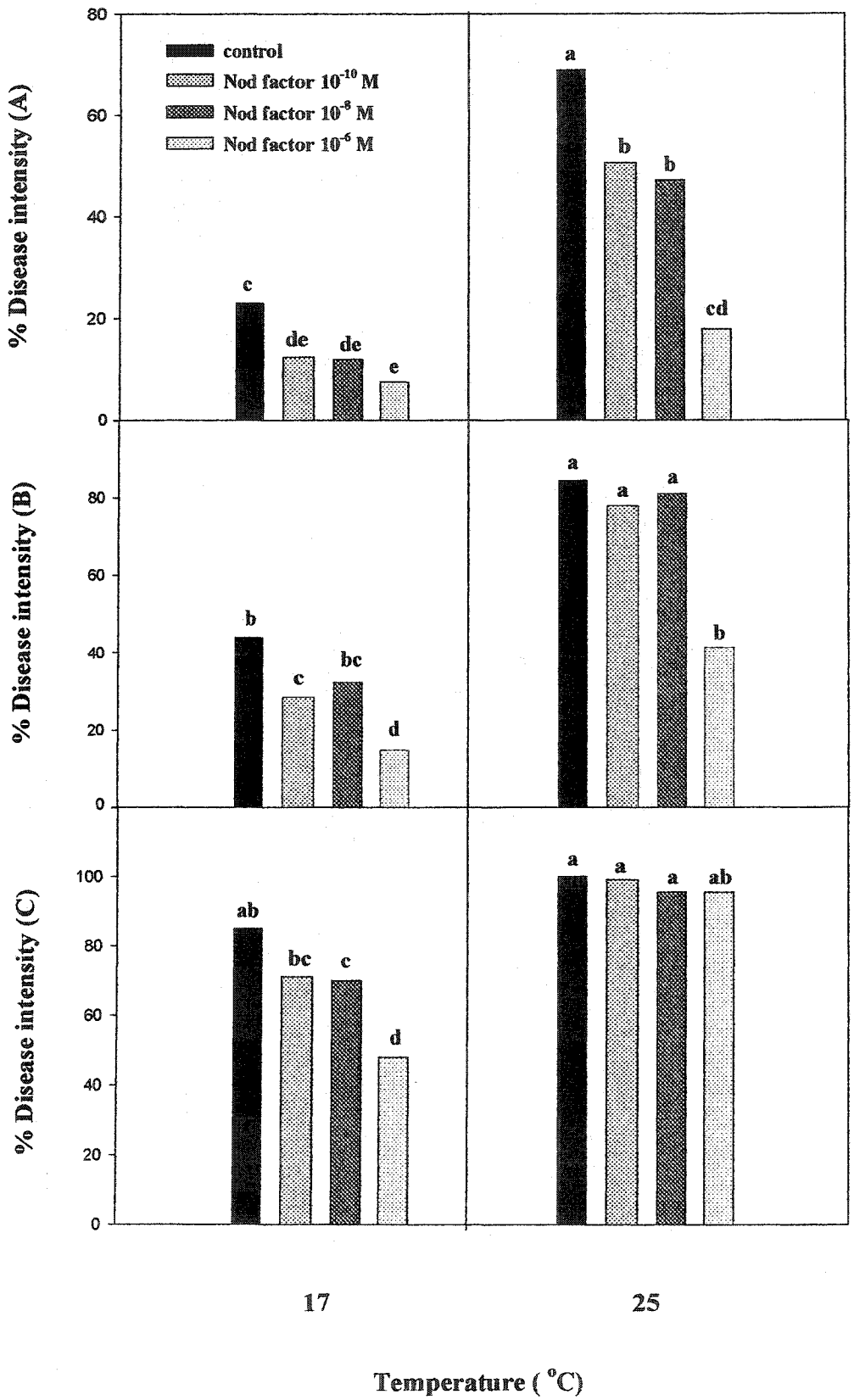
Nod factor also act as elicitors and provoke alkalinization and oxidative burst reactions in non-legume plants, similar to the response provoked by chitin fragments. Thus, it is reasonable to assume that Nod factor acted as an elicitor of plant defense reactions (Baier et al., 1999). Even though the presence of chitin as a structural unit of Nod factor suggests possible degradation of certain Nod factor by root hydrolytic

enzymes, such as chitinases, which are often associated with defense reactions against fungal attack, evidence presented by Staehelin et al. (1994; 2000) suggested that the certain Nod factor substitutions protect the compounds from inactivation by legume chitinases. Moreover, perception of substituted and non-substituted Nod factor by the host plant has been reported, suggesting "the existence of two signal perception systems, one transmitting the host-specific signal, the other representing an ancient reception system for a generic Nod factor structure" (Felle et al., 1996). However, in soybean roots and suspension cell cultures chitin-binding protein was identified in plasma membrane preparations; the purified Nod factor [Nod Bj-V ($C_{18:1}$, MeFuc)] acted as competitive inhibitor of chitin oligosaccharide binding to this protein, suggesting that soybean perceived both molecules by the same binding site, based on similarity of structural features, the chitin backbone for example, so that Nod factor might be anticipated to induce defense plant responses (Day et al., 2001). This finding supports, at least in part, the data presented in the current study. In addition, we have observed PAL induction by Nod factor, and PAL induction has also been reported for chitin oligomers (Khan et al., 2003).

It is known that sub-optimal RZTs negatively affect the growth rate of soybean (Zhang et al., 1995) so that plants growing under favorable RZTs exhibit faster growth. It is possible that this is, indirectly, the reason underlying the differential rates of disease progression observed at the two RZTs. Disease development was slower at the lower RZT. This is interesting as the disease developed on the leaves, which were at the same temperature for both RZTs, implying that the difference between the two RZTs was due to materials translocated from the roots to the shoots. The difference in disease

progression between the two RZTs was maintained, particularly at 10^{-6} M, throughout the experiment (Fig. 5. 3). It is known that the phenylpropanoid pathway, which starts with PAL, can be induced by abiotic stress factors, such as low temperature, and that levels of PAL induction can vary with the stage of plant development, genotype, and environmental conditions (Dixon and Paiva, 1995). Thus, it is reasonable to assume that the low RZT and Nod factor treatment might have an additive effect on plant defense reactions, through PAL induction. Our disease data suggest that PAL induction was greater or somehow more effective, at the lower RZT than at the higher one. It is not possible to show a correlation between PAL induction and disease incidence for two reasons, 1. the PAL assay was conducted under optimal temperature conditions, 2. Nod factor was applied to cut plants for PAL assay and to intact roots in the disease development work. It is worth noting that two types of infection, symbiotic (Zhang et al., 1995) and pathogenic, can be slowed by low RZT, while N_2 fixation occurs in the roots and the powdery mildew development is on the leaves, suggesting the presence of common factor in the plant effecting both types of infection, even if the infection is caused by a symbiont in one case and a pathogen in the other.

Fig. 5.1. The effect of Nod factor concentration and temperature on disease intensity on soybean leaves. A, B, and C represent data for weeks 1, 2, and 3. Bars associated with the same lower case letter are not significantly different, by an ANOVA (p = 0.05) protected $LSD_{0.05}$ test.


control
Nod factor 10-10 M
Nod factor 10-8 M
Nod factor 10-6 M
% Disease intensity (A)
% Disease intensity (B)
% Disease intensity (C)
0
20
40
60
80
100
c
de
de
e
a
b
b
cd
b
c
bc
d
a
a
a
b
ab
bc
c
d
a
a
a
ab
17
25
Temperature (°C)

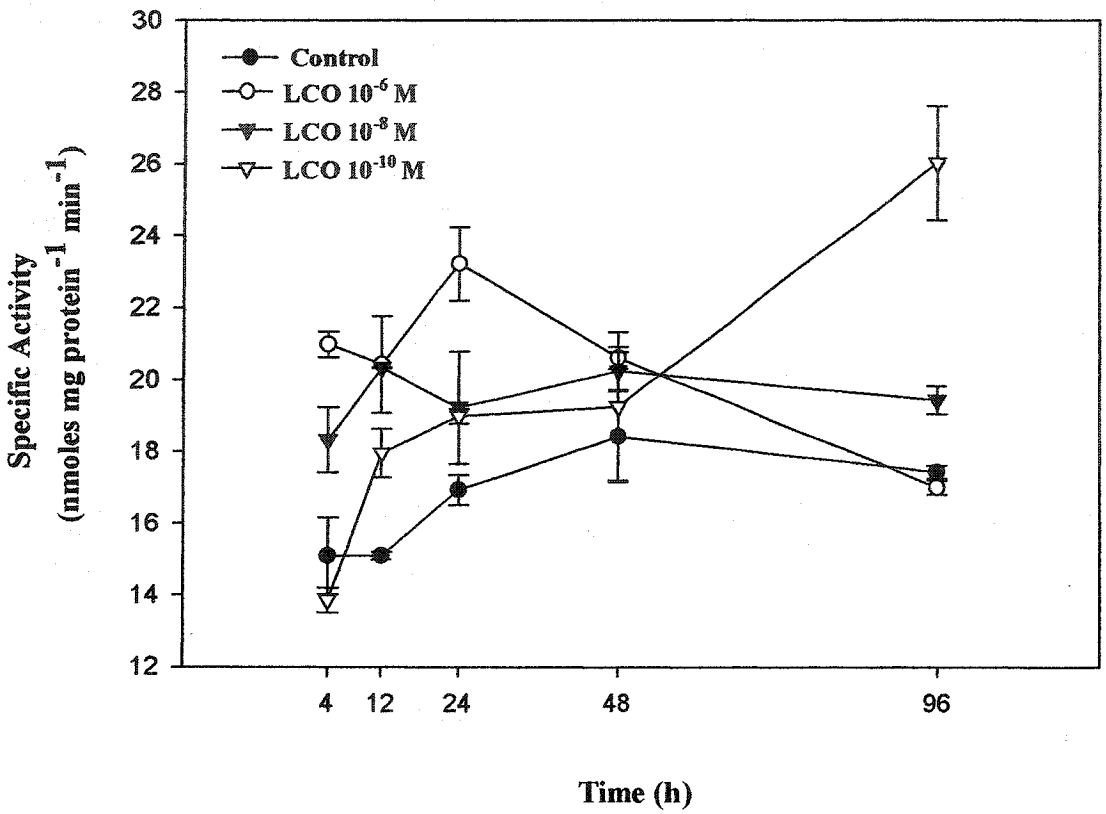


Fig. 5.2. Phenylalanine ammonia-lyase specific activity as function of time in soybean leaves treated with a range of Nod factor concentrations. Each value is plotted as the mean ± S.E. (n = 6)

Table 5.1. Effect of Nod factor treatment on the growth and development of powdery mildew on soybean leaves at 17 °C RZT.

Treatment *(M)	h after treatment	germination ave. (%)	Appresorium formation ave. (%)	Colony diameter ave.(μm)	No. of hyphal tubes / spore (ave.)
10^{-6}	4	5.2	*** -	-	-
10^{-8}	4	7.7	-	-	-
10^{-10}	4	5.6	-	-	-
0.0**	4	6.7	-	-	-
10^{-6}	12	11.7	70.5	-	-
10^{-8}	12	10.6	72.2	-	-
10^{-10}	12	14.0	66.6	-	-
0.0	12	14.6	68.8	-	-
10^{-6}	48	-	-	146.0 b	2.6 c
10^{-8}	48	-	-	192.2 a	3.1 ab
10^{-10}	48	-	-	190.5 a	3.0 b
0.0	48	-	-	202.5 a	3.3 a
10^{-6}	96	-	-	670.8 c	-
10^{-8}	96	-	-	744.2 c	-
10^{-10}	96	-	-	852.5 b	-
0.0	96	-	-	931.6 a	-

Values in the same column followed by the same letter are not significantly different by an ANOVA-protected $LSD_{0.05}$ test. * = Molar, ** = control, *** = not applicable.

Table 5.2. Effect of Nod factor treatment on the growth and development of powdery mildew on soybean leaves at 25 °C RZT.

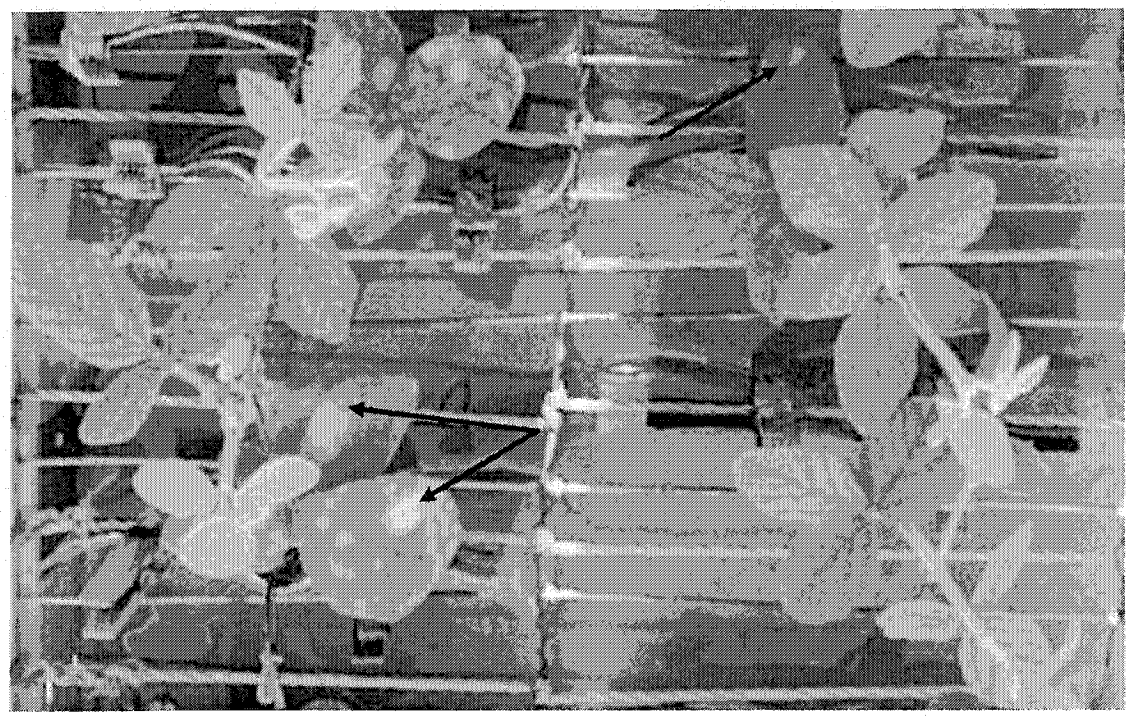
Treatment *(M)	h after treatment	germination ave. (%)	Appresorium formation ave. (%)	Colony diameter ave.(µm)	No.of hyphal tubes / spore (ave.)
10^{-6}	4	18.6	*** -	-	-
10^{-8}	4	22.2	-	-	-
10^{-10}	4	20.9	-	-	-
0.0**	4	20.5	-	-	-
10^{-6}	12	25.5	80.9 b	-	-
10^{-8}	12	24.3	84.5 b	-	-
10^{-10}	12	29.5	88.8 ab	-	-
0.0	12	29.5	92.5 a	-	-
10^{-6}	48	-	-	382.4 b	3.7 b
10^{-8}	48	-	-	404.5 b	3.8 ab
10^{-10}	48	-	-	418.2 ab	3.9 a
0.0	48	-	-	453.1 a	4.0 a

Values in the same column followed by the same letter are not significantly different by an ANOVA-protected $LSD_{0.05}$ test.

* = Molar.

** = Control.

*** = not applicable.



(A) Control **(B) Nod factor 10^{-6} M**

Fig. 5.3. Effect of Nod factor on disease incidence at 17 °C RZT. (A) Control without Nod factor treatment. (B) Nod factor treated soybean. Arrows show typical powdery mildew symptoms, irregular white patches on soybean leaves.

Chapter VI

General Discussion

Symbiosis, in the broadest sense, means organisms living together. This can be beneficial to both organisms, as in the rhizobia-legume N_2-fixation association, or detrimental to one organism, as in interactions between disease causing pathogenic microorganisms and host plants. In the case of plant-microbe relationships, the establishment of such relationships requires three conditions: an appropriate microorganism, an appropriate host plant, and favorable environmental conditions (Agrios, 1997). The outcome of the two types of interaction depends on recognition between the microbe and the plant; an early step leading to a phytopathogenic reaction, or to a compatible symbiotic rhizobia-legume association, in both cases, leading to infection.

Plants are able to recognize chitin fragments and these can act as elicitors of disease responses; these fragments are structural constituents of fungal cell walls (Ebel and Mithöfer, 1998). Similarly, the recognition step in the rhizobia-legume association requires coordinated exchange of specific signal molecules, generally flavonoids, excreted by legume root systems and Nod factor excreted by the micorsymbiont (Geurts and Bisseling, 2002). The presented research was designed and accomplished to investigate 1. the effect of suboptimal growth temperatures on the production of Nod factor by two strains of *B. japonicum*, 2. the interaction of abiotic stresses (low temperature, low pH and salinity) and Nod factor levels on the ability of soybean root hairs to respond to Nod factor form *B. japonicum*, and 3. the interaction of Nod factor and low RZT stress, on the ability of Nod factor treatments to induce resistance to the

development of a foliar disease, soybean powdery mildew, and the role PAL induction might play in this.

Suboptimal RZT exerts negative effects on the early stages of soybean nodule development, especially below 17 °C RZT. This has been related, at least in part, to reduced levels of genistein [the major soybean-to-*B. japonicum* signal (Zhang and Smith, 1994; Pan and Smith, 1998)]. Exposure of *B. japonicum* cells to genistein causes *nod* gene induction, leading to Nod factor synthesis, which plays a vital role in root hair deformation, causing pockets where rhizobia are entrapped during the early stages of nodulation. Thus, I tested the effects of three growth temperatures (two stressful and one optimal) on the production of Nod factor by two strains of *B. japonicum* commonly used in commercial inoculants under American and Canadian conditions.

In chapter three I showed that the greatest production, on a per cell basis, of the main Nod factor produced by *B. japonicum* occurred at an optimal growth temperature (28 °C), and this was observed for both of the tested *B. japonicum* strains, 532C and USDA110, while suboptimal incubation temperatures negatively affected the ability of both stains to produce Nod factor. Nod Bj-V ($C_{18:1}$, MeFuc) was first purified and identified as the main Nod factor produced by *B. japonicum* USDA110 by Sanjuan et al. (1992), and from 532C by Souleimanov et al. (2002). Work in our laboratory has shown that other Nod factor form *B. japonicum* 532C possess different length acyl side chains and different substitutions along the chitin backbone, similar to those isolated from cultures of *B. japonicum* strains USDA135 (Carlson et al., 1993).

As growth temperature decreased, progressive reductions in Nod factor production were observed, therefore we postulated that this might contribute to the already reported low RZT related reductions in soybean nodulation and delay in onset of nitrogen fixation (Zhang et al., 1995). The observed higher Nod factor production, both on total culture and per cell bases, as temperature increased to an optimum level (chapter three) indicates that at least part of the low RZT inhibition of soybean nodulation is caused by inhibition of Nod factor production, even when an appropriate level of genistein is present. The observation that strain 532C, which is known to be well adapted to the Canadian growth conditions (Hume and Shelp, 1990) produces more Nod factor than USDA110 at lower RZTs suggests a mechanism for its better performance in inoculants under cooler Canadian conditions.

When Nod factor is perceived by a legume root system, the first visible response is root hair deformation. The effect of three abiotic stresses (low temperature, low pH and salinity) on the ability of Nod factor to trigger this stage of the rhizobia-legume symbiosis establishment was the subject of chapter four. The greatest degree of root hair deformation (HAD) in response to Nod factor application was observed with the highest concentration (10^{-6} M) applied. This concentration has previously been shown to be effective at inducing HAD in the absence of abiotic stresses, and induction of specific defense-related responses, respectively (Savoure et al., 1997; Prithiviraj et al., 2000).

The inhibitory effect exerted by suboptimal growth temperature on Nod factor production (chapter three) was also observed for root hair deformation (chapter four). This response was concentration and incubation temperature dependant, and therefore, lower Nod factor bioactivity was observed at lower incubation temperatures. Low RZT inhibits early stages of symbiosis, particularly root hair deformation (Zhang and Smith, 1995). Since Nod factor, in conjunction with rhizobia, play a vital role in the root hair curling process (Lhuissier et al., 2001), and can even cause formation of anatomically complete nodules, on *Glycine soja,* in the absence of the microsymbiont (Stokkermans and Peters, 1994), and, given my data on HAD at low temperature (chapter four), it can be concluded that low rhizosphere temperatures negatively affects the bioactivity of Nod factor at the tested levels and contributes to inhibition of soybean nodulation at suboptimal RZTs.

The bioactivity of Nod factor was also evaluated under low pH stress conditions. Nod factor activity was concentration and stress-level dependant under low pH stress, with the lowest Nod factor bioactivity observed at pH 4. There was no interaction between Nod factor concentration and pH so that roots at low pH were not more affected by addition of Nod factor than those at high pH. Thus, perception of Nod factor is not specifically inhibited by low pH, but addition of higher levels of Nod factor could help overcome low pH inhibition of at least the first steps of soybean nodulation.

Salinity strongly inhibited soybean root hair deformation, and this was not improved by addition of higher levels of Nod factor. Thus, salinity may inhibit soybean

nodulation through specifically inhibiting the ability of soybean roots to respond to Nod factor, but adding additional Nod factor is unlikely to overcome this inhibition.

In these experiments a 1:1 mixture of two purified Nod factor from *B. japonicum* 532C were tested for their biological activity under three stress conditions (chapter four), each of which inhibited root hair deformation. The Nod factor solution caused root hair deformation, but, in general, at a lower frequency than when the main Nod factor was used alone, as seen in the data of Supanjani et al. (submitted). However, the intensity and distinctness of HAD was greater with two Nod factor than with one (chapter four). The published literature indicates that *B. japonicum* strains generally produce more than one biologically active Nod factor (Heidstra et al., 1994; Stokkermans et al., 1995), and that these work together to trigger root hair curling (Lhuissier et al., 2001). Nodulation in soybean *B. japonicum* is restricted to only part of the root system (Sadowsky et al., 1995) and it is generally the case that very large numbers of nodules are not formed. It may be that the lower frequency of root hair deformation resulting from a mixture of Nod factor (the natural condition) than a single Nod factor (Supanjani et al., submitted) is one of the mechanisms by which nodule number is controlled. The most frequently nodulated region of the root is located above the zone of rapid root elongation and below the smallest emergent root hairs present at the time of inoculation (Bhuvaneswari et al., 1980).

We attempted to mimic stress effects, *in vivo,* on early stages of Nod factor perception in the soybean-rhizobia association (chapter four). Because of the size of soybean seeds and the nature and number of treatments required we followed the method

described by Prithiviraj et al. (2000), a method proven to trigger a range of types and appreciable frequencies of soybean root hair deformation when compared to other methods (eg. Heidstra et al., 1994; Stokkermans et al., 1995) and the data from my controls showed that good frequencies of HAD were observed with this method.

We found that Nod factor induced soybean resistance to powdery mildew, caused by a biotrophic fungus with known effects on both photosynthesis and yield development (Dunleavy, 1979). It is worth noting that there are similar features between the two types of infection, symbiosis and disease, particularly the development of specialized interfaces by which nutrients are obtained. In the case of biotrophic fungi this interface is the haustoria, which surrounded by an invagination of plasma membrane, in the infected leaf cell (Harrison, 1999), and in the case of symbiotic infection, it is the bacteroid, the differentiated form of bacteria that fixes nitrogen, surrounded by plant derived membrane known as the peribacteroid membrane and located in root nodules (Schultze and Kondorosi, 1998; Geurts and Bisseling, 2002). In the latter case nutrient transfer is mutual (bidirectional), while in the pathogenic relationship it is unidirectional.

It is interesting that two types of soybean infection are controlled by a signal originating from the same microorganism (chapter five), and can be differentially recognized by the plant perception system with responses such as nodule formation or elicited pant defense responses inducing disease resistance. All Nod factor treatments reduced the incidence of disease on soybean leaves, with greater concentrations being more effective. The greatest inhibition and most persistent effect on disease progression was observed at the highest concentration of Nod factor (10^{-6} M) at both tested RZTs. It

is possible that this relatively high concentration caused a shift among biosynthetic pathways and triggered plant-defense responses against powdery mildew, as suggested by my PAL data. Schultze and Kondorosi (1998) postulated that the reported induction of defense-related plant reactions to Nod factor in the study of Savoure et al. (1997) was due to the high concentrations of Nod factor applied, that is, concentrations higher than the plants would normally see during a symbiotic infection. The expression of *nod* genes is tightly regulated, and thus specific types and amounts of Nod signal are required for symbiosis. An analogous observation was found with PAL specific activity. PAL is a key enzyme in phynelpropanoid pathway, through which a variety of phenolic compounds, such as phytoalexins, are produced. Nod factor, at 10^{-6} M, induced the accumulation of the phytoalexin medicarpin in *Medicago* cell cultures (Savoure et al., 1997). In chapter (five) Nod factor, at 10^{-6} M, induced the greatest PAL specific activity 24 h after treatment application, which is in accordance with the treatment effect on disease progression, though, after 96 h, PAL induction was detected following treatment with 10^{-10} M Nod factor. A possible explanation and consequences of this response was discussed in (chapter five).

Previous studies reported sensitivity of soybean to low RZTs, particularly during the early stages of nodulation (Zhang and Smith, 1994). I showed that the production of Nod factor and their perception by root hairs are also affected by low temperature (Chapters three and four). In chapter five the effects of exogenous Nod factor application to root systems on powdery mildew disease development was found to be Nod factor concentration and RZT dependant. In all cases the plant sensitivity and response to Nod factor was higher at low RZTs, and therefore, disease progression was lesser at low RZTs.

The low RZT plants developed more slowly and it may be that younger plants are more responsive to Nod factor treatment and more efficient in eliciting defense–related plant reactions than plants grown at optimal RZT. It is known that abiotic stresses can induce responses associated with biotic stresses, such as PAL induction, and it could also be that this allowed the low RZT plants to respond more quickly and/or to a greater degree than plants at an optimal RZT. It is not possible to directly compare PAL induction data with disease progression data, as the Nod factor was applied through cut soybean stems for the PAL work, and to intact root systems for the disease work, however, it seemed that the patterns of disease resistance and PAL activity, following Nod factor treatment, were consistent with each other.

Given the structural similarities between Nod factor (chitin-back bone) and oligochitin, elicitors of plant defense reactions, perhaps it should not be terribly surprising that Nod factor also elicits defense responses, such as PAL induction, leading to slower powdery mildew development. Recently, the induction of PAL activity in soybean leaves by chitin oligomers was reported (Khan et al., 2003). Further, in soybean roots and suspension cell cultures a chitin-binding protein has been identified in the plasma membrane for which the purified Nod factor [Nod Bj-V ($C_{18:1}$, MeFuc)] acts as competitive inhibitor of chitin oligosaccharides, suggesting that soybean perceived both molecules at the same binding site and based on similarity of structural features, a chitin backbone (Day et al., 2001), so that both would potentially induce defense responses. However, one must be cautious about making direct comparisons between modes of action for Nod factor and chitin oligomers based on similar chemical structure, particularly the chitin back-bone; modification of Nod factor structural features, such as

removal of sulfate, resulted in loss of their elicitor-like activity in *Medicago*. A similar loss of activity was also observed in Nod factor biological activity, suggesting the presence of a distinct perception system for Nod factor (Staehelin et al., 1994; Savoure et al., 1997). In addition, the amphipathic nature of Nod factor suggests an important role for the fatty acid moiety, perhaps allowing perception and insertion into the plasma membrane (Geurts and Bisseling, 2002) and protection against degradation by chitinases (Staehelin et al., 1994).

Over all, Nod factor at 10^{-6} M evoked the greatest frequency of root HAD in all experiments (chapter four) and the lowest frequency of powdery mildew symptom development, and induced the most rapid PAL activity increase suggesting some commonality among all of these responses.

Chapter VII

Acceptance or Rejection of Hypotheses and Contributions to Knowledge

The work conducted in the presented thesis is considered to contain original contributions to knowledge regarding the effects of suboptimal growth temperature on Nod factor production by *B. japonicum*, the ability of soybean root hairs to respond to Nod factor under conditions of abiotic stress, and the ability of Nod factor to induce disease resistance in soybean plants. The relevance of the findings to each of the initial hypotheses and the original nature of these findings are described here. For convenience, the hypotheses are restated now.

Hypothesis 1:

During the early stages of the soybean-*Bradyrhizobium* symbiosis establishment under Canadian growth conditions, low RZTs inhibit the ability of bacteria to produce nodulation signal molecules or "Nod factor", the *B. japonicum*-to-soybean-signal.

Hypothesis 2:

Low RZTs, low pH, and salinity reduce Nod factor bioactivity and response of soybean root hairs. Addition of higher concentrations of Nod signals potentially overcomes this inhibitory effect.

Hypothesis 3:

Addition of Nod factor to the soybean-bradyrhizobia system potentially induces plant defense responses against soybean powdery mildew.

Hypothesis 4:

Both, pathogenic infection (powdery mildew on soybean leaves) and controlled infection (symbiosis, root infection by rhizobia) are similarly affected by low RZTs.

Specific contributions to knowledge and acceptance of hypotheses:

1. The sensitivity of early stages of the soybean-rhizobia symbiosis establishment to low temperature is, at least in part, related to inhibition of Nod factor production by *B. japonicum* cells (chapter three, fig. 3.1). Thus, we accept hypothesis 1.

2. Exogenous addition of higher levels of Nod factor can help overcome low pH and low temperature inhibitions of root hair deformation associated with symbiotic infection of soybean by *B. japonicum* (chapter four, figs. 4.1 and 4.2). Thus, we accept hypothesis 2.

3. Exogenous additions of higher levels of Nod factor do not help to overcome the inhibition of soybean root hair deformations due to salinity stress (chapter four, fig. 4.3). Thus, we reject hypothesis 2.

4. At concentrations 10^{-6}, 10^{-8}, and 10^{-10} M, Nod Bj-V ($C_{18:1}$, MeFuc), reduced the incidence of soybean powdery mildew (chapter five, figs. 5.1, 5.3). This is the first report to address induction of soybean resistance to powdery mildew by Nod factor application. Thus we accept hypothesis 3.

5. Application of Nod factor to soybean plants induces PAL activity in a dose and time dependant fashion; this has not been previously reported (chapter five, fig. 5.2). Thus we accept hypothesis 3.

6. This is the first report to address the relationship between root zone temperature and disease progression (chapter five, fig. 5.1 A). Thus we accept hypothesis 4.

Chapter VIII

Suggestions for Future Research

Additional research that can be conducted to extend the work included in this thesis is as follows:

1. In this thesis I looked at the effects of low temperature on the production of Nod factor by *B. japonicum.* Additional work should be conducted to evaluate Nod factor production under other types of abiotic stress.

2. In the work I conducted I used only one concentration of genistein to induce the *nod* genes of *B. japonicum* cells under stress conditions. Research should be conducted to evaluate production of Nod factor under abiotic stresses as a function of the concentration of *nod* gene inducer.

3. I conducted research on the effects of suboptimal growth temperatures on the production of the Nod factor most abundantly produced by *B. japonicum.* It would be very useful to examine the effect of suboptimal growth temperature on the production of the other Nod factor produced by *B. japonicum*, and the relative abundance of these Nod factor to each other.

4. In this thesis I report research on the effects of low temperature on production of Nod factor by *B. japonicum.* In my case, "production" includes all aspects of synthesis and excretion as I measured the concentration of Nod factor in the resulting bacterial culture

medium. There is a need to determine the effect of suboptimal growth temperature on the excretion, vs. synthesis, of Nod factor.

5. I was able to show that, under some stress conditions, increasing the concentration of Nod factor increased the degree of root hair response. However, in many cases, the highest rate of response occurred at the highest level of added Nod factor. It would be instructive to determine the effects of higher concentrations of Nod factor on biological activity (root hair deformation) under the environmental stress conditions included this research.

6. I was able to show that addition of Nod factor, under controlled environment conditions, improved the first steps of the nodulation process when soybean root systems were stressed. Field research should be conducted to test of the ability of Nod factor to overcome inhibition of soybean nodulation by various environmental stresses.

7. My work demonstrated that Nod factor deterred the development of powdery mildew on soybean. There is a need to determine the mechanisms underlying this induced disease resistance, including identification and characterization of the compounds, such as phytoalexins, involved in Nod factor induced defense reactions in soybean plants.

8. I was able to show that Nod factor treatment slowed the development of powdery mildew on soybean. This work should be extended to include the effects of Nod factor application on other soybean diseases of economic importance.

9. The work conducted in this thesis has focused entirely on soybean. It would be useful to test my findings on other crop legume species and, in the case of the disease control work, beyond the legume family.

References

Agrios, G. N. 1997. Plant Pathology, 4th Ed. Academic Press, San Diego, USA. 635p.

Alva, A.K., Edwards, D.G., Asher, C.J., and Suthipradit, S. 1987. Effects of acid soil infertility factors on growth and nodulation of soybean. *Agronomy Journal*. 79: 302-306.

Appelbaum, E. 1990. The *Rhizobium/Bradyrhizobium*-legume symbiosis. In P.M. Gresshof, ed., *Molecular Biology of Symbiotic Nitrogen Fixation*, pp. 131-158. CRC Press, Boca Raton, FL.

Baier, R., Schiene, K., Kohring, B., Flaschel, E., and Niehaus, K. 1999. Alfalfa and tobacco cells react differently to chitin oligosaccharides and *Sinorhizobium meliloti* nodulation factors. *Planta*. 210: 157-164.

Baron, C., and Zambryski, P.C. 1995. The plant response in pathogenesis, symbiosis, and wounding: variations on a common theme? *Annual Review of Genetics*. 29: 107-129.

Beaudoin-Eagan, L.D., and Thorpe T.A. 1985. Tyrosine and phenylalanine ammonia lyase activities during shoot initiation in tobacco callus cultures. *Plant Physiology*. 78: 438-441.

Bhuvaneswari, T.V., Goodman, R.N., and Bauer, W.D. 1980. Early events in the infection of soybean [*Glycine max* (L.) Merr.] by *Rhizobium japonicum*. I. Location of infectible root cell. *Plant Physiology*. 66: 1027-1031.

Boller, T. 1995. Chemoperception of microbial signals in plant cells. *Annual Review of Plant Physiology and Plant Molecular Biology*. 46: 189-214.

Bordeleau, L.M., and Prevost, D. 1994. Nodulation and nitrogen fixation in extreme environments. *Plant and Soil*. 161: 115-125.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemisrty*. 72: 248-254.

Buonassissi, A.J., Copeman, R.J., Pepin, H.S., and Eaton, G.W. 1986. Effect of *Rhizobium* spp. on *Fusarium* f. sp. *phaseoli*. *Canadian Journal of Plant Pathology*. 8: 140–146.

Carlson, R.W., Sanjuan, J., Bhat, R., Glushka, J., Spaink, H.P., Wijfjes, A.H.W., van Brussel, A.N.A., Stokkermans, T.J.W., Peters, N.K., and Stacey, G. 1993. The structures and biological activities of the lipo-oligosacchride nodulation signals produce by type I and II strains of *Bradyrhizoboium japonicum*. *Journal of Biological Chemistry*. 268: 18372-18381.

Carver, T.L.W., and Adaigbe, M. E. 1990. Effects of oat host genotype, leaf age and position and incubation humidity on germination and germling development by *Erysiphe graminis* f.sp. *avenae*. *Mycological Research.* 94: 18-26.

Cho, M.J., and Harper, J. 1991. Effect of inoculation and nitrogen on isoflavonoid concentration in wild-type and nodulation-mutant soybean roots. *Plant Physiology*. 95: 435-442.

Conçalves, E.C.P., and Di Mauro, A.O. 2002. Genetics of resistance to powdery mildew (*Microsphaera diffusa*) in Brazilian soybean populations. *Genetics and Molecular Biology*. 25: 339-342.

Cordovilla, M.D.P, Ligero, F., and Lluch, C. 1999. Effect of salinity on growth, nodulation and nitrogen assimilation in nodules of faba bean (*Vicia faba* L.). *Applied Soil Ecology*. 11: 1-7.

Cullimore, J.V., Ranjeva, R., and Bono, J.J. 2001. Perception of lipo-chitooligosaccharide Nod factors in legumes. *Trends in Plant Science*. 6: 24-30.

Dakora, F.D. 2003. Defining new roles for plant and rhizobial molecules in sole and mixed plant cultures involving symbiotic legumes. *New Phytologist*. 158: 39-49.

Dakora, F.D., Joseph, C.M., and Phillips, D.A. 1993. Alfalfa (*Medicago sativa* L.) root exudates contain isoflavonoids in the presence of *Rhizobium meliloti*. *Plant Physiology*. 101: 819-824.

Dart, P., Day, J., Islam, R., and Dobereiner, J. 1976. Symbiosis in tropical grain legumes: some effects of temperature and the composition of the rooting medium. In P.S. Nutman, ed., *Symbiotic Nitrogen Fixation in Plants*, pp. 361-383. Camb. Univ. Press, Cambridge.

Day, R.B., Okada, M., Ito, Y., Tsukada, K., Zaghouani, H., Shibuya, N., and Stacey, G. 2001. Binding site for chitin oligosaccharides in the soybean plasma membrane. *Plant Physiology*. 126: 1162-1173.

Denarie, J., Debelle, F., and Prome, J.-C. 1996. *Rhizobium* lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. *Annual Review of Biochemistry*. 65: 503-535.

Denarie, J., Debelle, F., and Rosenberg, C. 1992. Signaling and host range variation in nodulation. *Annual Review of Microbiology*. 46: 497-531.

Dixon, R.A., and Paiva, N.L. 1995. Stress-induced phenylpropanoid metabolism. *The Plant Cell*. 7: 1085-1097.

Dixon, R.A., Harrison, M.J., and Paive, N.L. 1995. The isoflavonoid phytoalexins pathway: from enzymes to genes to transcription factors. *Physiologia Plantarum*. 93: 385-392.

Djordjevic, M.A., Schofield, P.R., Ridge, R.W., Morrison, N.A., Bassam, B.J., Plazinski, J., Watson, J.M., and Rolfe, B.G. 1985. *Rhizobium* nodulation genes involved in root hairs curling (Hac) are functionally conserved. *Plant Molecular Biology*. 4: 147-160.

Dong, X. 1998. SA, JA, ethylene, and disease resistance in plants. *Current Opinion in Plant Biology*. 1: 316-323.

Dunleavy, J.M., 1979. Soybean seed yield losses caused by powdery mildew. *Crop Science*. 18: 337-339.

Dyachok, J.V., Tobin, A.E., Price, N.P.J., and von Arnold, S. 2000. Rhizobial Nod factors stimulate somatic embryo development in *Picea abies*. *Plant Cell Reporters*. 19: 290–297.

Dyachok, J.V., Wiweger, M., Kenne, L., and von Arnold, S. 2002. Endogenous Nod factor-Like signal molecules promote early somatic embryo development in Norway spruce. *Plant Physiology*. 128: 523–533.

Eardly, B.D., Hannaway, D.B., and Bottomley, P.J. 1984. Nitrogen nutrition and yield of seedling alfalfa as affected by ammonium nitrate fertilization. *Agronomy Journal.* 77: 57-62.

Ebel, J., and Mithöfer, A. 1998. Early events in the elicitation of plant defence. *Planta.* 206: 335-348.

Ehrhardt, D.W., Atkinson, E.M., and Long, S.R. 1992. Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. *Science*. 256: 998-1000.

Ehrhardt, D.W., Wais, R., and Long, S.R. 1996. Calcium spiking in plant root hairs responding to *rhizobium* nodulation signals. *Cell.* 85: 673-681.

Ehteshamul-Haque, and S., Ghaffar, A. 1993. Use of rhizobia in the control of root rot diseases of sunflower, okra, soybean and mungbean. *Journal of Phytopathology.* 138: 157–163.

Ek-Jander, J., and Fahraeus, G. 1971. Adaptation of *Rhizobium* to subarctic environment in Scandinavia. *Plant and Soil.* (special vol.) 129-137.

Elad, Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop protection.* 19: 709-714.

Elsheikh, E.A.E. 1998. Effects of salt on rhizobia and bradyrhizobia: a review. *Annals of Applied Biology*. 132: 507-524.

Elsheikh, E.A.E. and Wood, M. 1995. Nodulation and N2 fixation by soybean inoculated with salt-tolerant rhizobia or salt-sensitive bradyrhizobia in saline soil. *Soil Biology and Biochemistry*. 27: 657-661.

Estabrook, E.M., and Gopalan, C.S. 1991. Differential expression of phenylalanine ammonia-lyase and chalcone synthase during soybean nodule development. *The Plant cell*. 3: 299-308.

Evans, L.S., Lewin, K.F., and Vella, F.A. 1980. Effect of nutrient medium pH on symbiotic nitrogen fixation by *Rhizobium leguminosarum* and *Pisum sativum*. *Plant and Soil*. 56: 71-80.

Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M. 2000. How alfalfa root hairs discriminate between nod factors and oligochitin elicitors. *Plant Physiology*. 124: 1373-1380.

Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M. 1996. Rapid alkalinization in alfalfa root hairs in response to rhizobial lipochitooligosaccharide signals. *The Plant Journal*. 10: 295-301.

Firmin, J., Wilson, K., Carlson, R.W, Davies, A., and Downie, J. 1993. Resistance to nodulation of cv. Afghanistan peas is overcome by nodX, which mediates an *O*-acylation of the *Rhizobium leguminosarum* lipo-oligosaccharide nodulation factor. *Molecular Microbiology.* 10: 351-360.

Fofana, B., McNally, D.J., Labbe`, C., Boulanger, R., Benhamou, N., Seguin, A., and Belanger, R.R. 2002. Milsana-induced resistance in powdery mildew-infected cucumber plants correlates with the induction of chalcone synthase and chalcone isomerase. *Physiological and Molecular Plant Pathology.* 61: 121-132.

Frey, S., and Carver, T.L. W. 1998. Induction of systemic resistance in pea to pea powdery mildew by exogenous application of salicylic acid. *Journal of Phytopathology.* 146: 239-245.

Fujihara, S., and Yoneyama, T. 1993. Effects of pH and osmotic stress on cellular polyamine contents in the soybean *Rhizobium fredii* P220 and *Bradyrhizobium japonicum* P1017. *Applied and Environmental Microbiology.* 59: 1104-1109.

Fyson, A., and Sprent, J.I. 1982. The development of primary root nodule on *Vicia faba* L. grown at two root temperatures. *Annals of Botany.* 50: 681-692.

Garcia, M.L., Dunlap, J., Loh, J., and Satcey, G. 1996. Phenotypic characterization and regulation of the nolA gene of *Bradyrhizobium japonicum*. *Molecular Plant-Microbe Interactions.* 9: 625-635.

Geurts, R., and Bisseling, T. 2002. *Rhizobium* Nod factor perception and signaling. *The Plant Cell.* (special vol.) 239-249.

Gibson, A.H. 1971. Factors in the physical and biological environment affecting nodulation and nitrogen fixation by legumes. *Plant and Soil.* (special vol.) 139-152.

Grag, B.K., and Gupta, I.C. 2000. Nodulation and symbiotic nitrogen fixation under salt stress. *Current Agriculture.* 24: 23-35.

Hammond-Kosack, K.E., and Jones, J.D.G. 1996. Resistance gene-dependent plant defense responses. *The Plant Cell.* 8: 1773-1791.

Harrison, M.J. 1999. Biotrophic interfaces and nutrient transport in plant/fungal symbiosis. *Journal of Experimental Botany.* 50: 1013-1022.

Hartog, M.D., Musgrave, A., and Munnik, T. 2001. Nod factor-induced phosphatidic acid and diacylglycerol pyrophosphate formation: a role for phospholipase C and D in root hair deformation. *The Plant Journal.* 25: 55-65.

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

Heitefuss, R. 2001. Defence reactions of plants to fungal pathogens: principles and perspectives, using powdery mildew on cereals as an example. *Naturwissenschaften*. 88: 273-283.

Hicks, D.R. 1978. Growth and development. In G.A. Norman, ed., *Soybean Physiology, Agronomy, and Utilization*, pp. 17-41. Academic Press, New York, NY.

Hirsh, A.M., Lum, M.R., and Downie, J.A. 2001. What makes the *Rhizobia*-legume symbiosis so special? *Plant Physiology*. 127: 1484-1492.

Horner, H.T., Healy, R.A., Cervantes-Martinez, T. and Palmer, R.G. 2003. Floral nectary fine structure and development in *Glycine max* L. (Fabaceae). *International Journal of Plant Sciences*. 164: 675-690.

Huang, C.Y., Boyer, J.S., and Vanderhoef, L.N. 1975. Limitation of acetylene reduction (nitrogen fixation) by photosynthesis in soybean having low water potentials. *Plant Physiology*. 56: 228- 232.

Hume, D.J., and Shelp, B.J. 1990. Superior performance of the Hup *Bradyrhizobium japonicum* strain 532C in Ontario soybean field trails. *Canadian Journal of Plant Science*. 70: 661-666.

Hungria, M., and Stacey, G. 1997. Molecular signals exchanged between host plants and rhizobia: Basic aspects and potential application in agriculture. *Soil Biology and Biochemistry*. 29: 819-830.

Hungria, M., and Vargas, M.A.T. 2000. Environmental factors affecting N2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field Crops Research*. 65: 151-164.

Ikeda, J. 1994. The effect of short term withdrawal of NaCl stress on nodulation of white clover. *Plant and Soil.* 158: 23-27.

Ip, H., D'Aoust, F., Begum, A.A., Zhang, H., Smith, D.L., Driscoll, B.T., and Charles, T.C. 2001. *Bradyrhizobium japonicum* mutant with enhanced sensitivity to genistein resulting in altered nod gene regulation. *Molecular Plant-Microbe Interactions.* 14: 1404-1410.

Jones, F.R., and Tisdale, W.B. 1921. Effect of soil temperature upon the development of nodules on the roots of certain legumes. *Journal of Agricultural Research*. 22: 17-37.

Kelly, M.N., and Irving, H.R. 2002. Nod factors activate both heterotrimeric and monomeric G-proteins in *Vigna unguilata* (L.) Walp. *Planta*. 216: 674-685.

Khan, W.M., Prithiviraj, B., and Smith, D.L. Chitosan and chitin oligomers increase phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities in soybean leaves. *Journal of Plant Physiology*. 160: 859-863.

Kosslak, R.M., Bookland, R., Barkei, J., Paaren, H., and Appelbaum, E.R. 1987. Induction of *Bradyrhizobium japonicum* common *nod genes* by isoflavones isolated from *Glycine max*. *Proceedings of the National Academy of Sciences of the United States of America*. 84: 7428-7432.

Krishnan, H.B., and Pueppke, S.G. 1998. Genetic characterization of a mutant of *Sinorhizobium fredii* strain USDA208 with enhanced competitive ability for nodulation of soybean, *Glycine max* (L.) Merr. *FEMS Microbiology Letters*. 165: 215-220.

Krishnan, H.B., Lorio, J., Kim, W.S., Jiang, G.Q., Kim, K.Y., DeBoer, M., and Pueppke, S.G. 2003. Extracellular proteins involved in soybean cultivar-specific nodulation are associated with pilus-like surface appendages and exported by a type III protein secretion system in *Sinorhizobium fredii* USDA257. *Molecular Plant-Microbe Interactions*. 16: 617-625.

Layzell, D.B., Rochmann, P., and Canvin, D.T. 1984. Low temperatures and nitrogenase activity in soybean. *Canadian Journal of Botany*. 62: 965-971.

Lazarwitz, S.G., and Bisseling, T. 1997. Plant development from the cellular perspective: Integrating the signals. *The Plant Cell*. 9: 1884-1900.

Lehman, S.G., 1931. Powdery mildew of soybean. *Journal of the Elisha Mitchell Scientific Society*. 46: 190-195.

Lhuissier, F.G.P., De Ruijter, N.C.A., Sieberer, B.J., Esseling, J.J., and Emons, A.M.C. 2001. Time course of cell biological events evoked in legume root hairs by *Rhizobium* Nod factors: State of the art. *Annals of Botany*. 87: 289-302.

Lie, T.A. 1981. Environmental physiology of the legume-*Rhizobium* symbiosis. In W.J. Broughton, ed., *Nitrogen Fixation Volume* 1. *Ecology*, pp. 104-134. Clarendon Press, Oxford.

Lindemann, W.C., and Ham, G.E. 1979. Soybean plant growth, nodulation, and nitrogen fixation as affected by root temperature. *Soil Science Society of America Journal.* 43: 1134-1137.

Loh, J.T., Yuen-Tsai, J.P.-Y., Stacey, M.G., Lohar, D., Welborn, A., and Stacey, G. 2001. Population density-dependent regulation of the *Bradyrhizobium japonicum* nodulation genes. *Molecular Microbiology*. 42: 37-46.

Lohnes, D.G., and Nickell, C.D. 1994. Effects of powdery mildew alleles *Rmd-c*, *Rmd*, and *rmd* on yield and other characteristics in soybean. *Plant Disease*. 78: 299-301.

Lynch, D.H., and Smith, D.L. 1993. Soybean (*Glycine max (L.) Merr*) nodulation and N_2 fixation as affected by period of exposure to a low root zone temperature. *Physiologia Plantarum*. 88: 212-220.

Marie, C., Deakin, W.J., Viprey, V., Kopcinska, J., Golinowski, W., Krishnan, H.B., Perret, X., and Broughton, W.J. 2003. Characterization of Nops, nodulation outer proteins, secreted via the type III secretion system of NGR234. *Molecular Plant-Microbe Interactions*. 16: 743-751.

Martinez-Abarca F., Herrera-Cervera J.A., Bueno, P., Sanjuan, J., Bisseling, T., and Olivares, J. 1998. Involvement of salicylic acid in the establishment of the *Rhizobium meliloti*-alfalfa symbiosis. *Molecular Plant-Microbe Interactions*. 11: 153-155.

McClary, D. 1997. The role of Ontario soybean growers marketing board in soybean research in Ontario. Research Committee Chair, Ontario Soybean Growers Marketing Board.

McKay, I.A., and Djordjevic, M.A. 1993. Production and excretion of nod metabolites by *Rhizobium leguminosarum* bv. trifolii are disrupted by the same environmental factors that reduce nodulation in the field. *Applied and Environmental Microbiology*. 59: 3385-3392.

McLaughlin, S.B., and Wimmer, R. 1999. Calcium physiology and terrestrial ecosystem processes. *New Phytologist*. 142: 373-417.

Mergaert, P., Van Montagu, M., and Holsters, M. 1997. Molecular mechanisms of Nod factor diversity. *Molecular Microbiology*. 25: 811-817.

Mergaert, P., van Montagu, M., Prome, J.C., and Holsters, M. 1993. Three unusual modifications, a *D*-arabinosyl, an *N*-methyl. and a carbamoyl group, are present on the Nod factors of *Azorhizobium caulinodans* strain ORS571. *Proceedings of the National Academy of Sciences of the United States of America.* 9: 1551-1555.

Mignucci, J.S., and Boyer, S.J. 1979. Inhibition of photosynthesis and transpiration in soybean infected by *Microsphaera diffusa*. *Phytopathology*. 69: 227-230.

Minami, E., Kouchi, H., Carlson, R., Cohn, J., Kolli, V., Day, R., Ogawa, T., and Stacey, G. 1996. Cooperative action of lipo-chitin nodulation signals on the induction of the early nodulin, ENOD2, in soybean roots. *Molecular Plant-Microbe Interactions*. 9: 574-583.

Minami, E., Kouchi, H., Cohn, J.R., Ogawa, T., and Stacey, G. 1996. Expression of early nodulin, ENOD40, in soybean roots in response to various lipo-chitin signal molecules. *The Plant Journal*. 10: 23-32.

Mithöfer, A., Ebel, J., Bhagwat, A.A., Boller, T., and Neuhaus-Url, G. 1999. Transgenic aequorin monitors cytosolic calcium transients in soybean cells challenged with b-glucan or chitin elicitors. *Planta*. 207: 566-574.

Morrison, T.A., and Buxton, D.R. 1993. Activity of phenylalanine ammonia-lyase, tyrosine ammonia-lyase, and cinnamyl alcohol dehydrogenase in the maize stalks. *Crop Science*. 33: 1264-1268.

Müller, J., Staehelin, C., Xie, Z-P., Neuhaus-Url, G., and Boller, T. 2000. Nod factors and chitooligomers elicit an Increase in cytosolic calcium in aequorin-expressing soybean cells. *Plant Physiology*. 124: 733–739.

Munns, D.N. 1968. Nodulation of *Medicago sativa* in solution culture. I. Acid-sensitive steps. *Plant and Soil*. 28: 129-146.

Ndakidemi, P.A., Dakora, F.D. 2003. Legume seed flavonoids and nitrogenous metabolites as signals and protectants in early seedling development. *Functional-Plant Biology*. 30: 729-745.

Ohashi, Y., Oka, A., Rodrigues-Pousada, R., Possenti, M., Ruberti, I., Morelli, G., and Aoyama, T. 2003. Modulation of phospholipid signaling by GLABRA2 in root-hair pattern formation. *Science*. 300: 1427-1430.

Pan, B., and Smith, D.L. 1998. Genistein and daidzein concentrations and contents in seedling roots of three soybean cultivars grown under three root zone temperatures. *Journal of Agronomy and Crop Science*. 180: 77-82.

Phillips, D.A., and Kapulnik, Y. 1995. Plant isoflavonoids, pathogens and symbionts. *Trends In Microbiology*. 3: 58-64.

Phillips, D.A., Wery, J., Joseph, C., Jones, A.D., and Teuber, L.R. 1995. Release of flavonoids and betaines from seeds of seven *Medicago* Species. *Crop Science*. 35: 805-808.

Phillips, D.A., Dakora, F.D., Sande, E., Joseph, C.M., and Zon, J. 1994. Synthesis, release, and transmission of alfalfa signals to rhizobial symbionts. *Plant and Soil*. 161: 69-80.

Phillips, D.V. 1984. Stability of *Microsphaera diffusa* and the effect of powdery mildew on yield of soybean. *Plant Disease*. 68: 953-956.

Price, N.P.J., Relic, B., Talmont, F., Lewin, A., Prome, D., Pueppke, S.G., Maillet, F., Denarie, J., Prome, J.C., and Broughton, W.J. 1992. Broad-host-range *Rhizobium* species strain NGR234 secretes a family of cabamoylated, and fucosylated, nodulation signals that are *O*-acetylated and sulphated. *Molecular Microbiology*. 6: 3575-3584.

Price, N.P.J. 1999. Carbohydrate determinants of *Rhizobium*-legume symbioses. *Carbohydrate Research*. 317: 1-9.

Prithiviraj, B., Souleimanov, A., Zhou, X., and Smith, D.L. 2000. Differential response of soybean (*Glycine max* (L.) Merr.) genotypes to lipo-chito-oligosaccharide Nod Bj-V ($C_{18:1}$, MeFuc). *Journal of Experimental Botany.* 51: 2045-2051.

Relic, B., Talmont, F., Kopcinska, J., Golinowski, W., Prome, J-C., and Broughton, W.J. 1993. Biological activity of *Rhizobium sp.* NGR234 Nod-factors on *Macroptilium atropurpureum. Molecular Plant-Microbe Interactions.* 6: 764-774.

Richardson, A.E., Djordjevic, M.A., Rolfe, B.G., and Simpson, R.J., 1988a. Effects of pH, Ca and Al on the exudation from clover seedlings of compounds that induce the expression of nodulation genes in *Rhizobium trifolii. Plant and Soil.* 109: 37-47.

Richardson, A.E., Simpson, R.J., Djordjevic, M.A., and Rolfe, B.G. 1988b. Expression of nodulation genes in *Rhizobium leguminosarum* biovar *trifolii* is affected by low pH and by Ca and Al ions. *Applied and Environmental Microbiology.* 54: 2541-2548.

Saadallah, K., Drevon, J.J., and Abdelly, C. 2001. Nodulation and growth of nodules in the common bean (*Phaseolus vulgaris*) under salt stress. *Agronomie.* 21: 627-634.

Sanjuan, J., Carlson, R.W., Spaink, H.P., Bhat, U.R., Barbour, W.M., Glushka, J., and Stacey, G. 1992. A *2-O*-methylfucose moiety is present in the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum. Proceedings of the National Academy of Sciences of the United States of America.* 89: 8789-8793.

SAS Institute Inc. 1989. *SAS users Guide*. Statistical Analysis Institute Inc., Cary, North Carolina. 1675 p.

Savoure, A, Sallaud, C., EL-Turk, J., Zuanazzi, J., Ratet, P., Schultze, M., Kondorosi, A., Esnault, R., and Konodorosi, E., 1997. Distinct response of *Medicago* suspension cultures and roots to Nod factors and chitin oligomers in the elicitation of defense-related responses. *The Plant Journal*. 11: 277-287.

Schmidt, P.E., Broughton, W.J., and Werner, D. 1994. Nod factors of *Bradyrhizobium japonicum* and *Rhizobium* sp. NGR234 induce flavonoid accumulation in soybean root exudates. *Molecular Plant-Microbe Interactions*. 7: 384-390.

Schultze, M., and Kondorosi, A. 1998. Regulation of symbiotic root nodule development. *Annual Review of Genetics*. 32: 33-57.

Sinclair, J., and Backman, P., 1989. Compendium of soybean diseases, 3rd edition, APS Press, The American Phytopathological Society. 106p.

Singh, U.P., and Prithiviraj, B. 1997. Neemazal, a product of neem (*Azadirachta indica*), induces resistance in pea (*Pisum sativum*) against *Erysiphe pisi*. *Physiological and Molecular Plant Pathology*. 51: 181-194.

Singleton, P.W., and Bohlool, B.B. 1984. Effect of salinity on the nodule formation by soybean. *Plant Physiology*. 74: 72-76.

Smith, D.L., and Hume, D.J. 1987. Comparison of assay methods for nitrogen fixation utilizing white bean and soybean. *Canadian Journal of Plant Science*. 67: 11-19.

Smith, P.H., Foster, E.M., Boyd, L.A., and Brown, J.K.M. 1996. The early development of *Erysiphe pisi* on *Pisum sativum* L. *Plant Pathology*. 45: 302-309.

Soedarjo, M., and Borthakur, D. 1998. Mimosine, a toxin produced by the tree-legume *Leucaena* provides a nodulation competition advantage to mimosine-degrading *Rhizobium* strains. *Soil Biology and Biochemistry*. 30: 1605-1613.

Somasegaran, P., and Hoben, H.J. 1994. Handbook for Rhizobia. Springer Laboratory. 449p.

Souleimanov A, Prithiviraj B, and Smith D.L. 2002. The major Nod factor of *Bradyrhizobium japonicum* promotes early growth of soybean and corn. *Journal of Experimental Botany*. 53: 1929-1934.

Souleimanov, A., Prithiviraj, B., Carlson, R.W., Jeyaretnam, B., and Smith D.L. 2002. Isolation and characterization of the major Nod factor of *Bradyrhizobium japonicum* strain 532C. *Microbiological Research*. 157: 25-28.

Spaink, H.P., Aarts, A., Stacey, G., Bloemberg, G.V., Lugtenberg, B.J.J., and Kennedy, E.P. 1992. Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod

metabolites using thin layer chromatography. *Molecular Plant-Microbe Interactions.* 5: 72-80.

Sprent, J.I. 1979. The Biology of Nitrogen Fixation. McGraw-Hill Book Company Ltd. Co., New York, NY. 196p.

Staehelin, C., Granado, J., Müller, J., Wiemken, A., Mellor, R.B., Felix, G., Regenass, M., Broughton, W.J., and Boller, T. 1994. Perception of *Rhizobium* nodulation factors by tomato cells and inactivation by root chitinases. *Proceedings of the National Academy of Sciences of the United States of America.* 91: 2196-2200.

Staehelin, C., Schultze, M., Tokuyasu, K., Poinsot, V., Promé, J-C., Kondorosi, E., and Kondorosi, A. 2000. N-deacetylation of *Sinorhizobium meliloti* Nod factors increases their stability in the *Medicago* sativa rhizosphere and decreases their biological activity. *Molecular Plant-Microbe Interactions.* 13: 72-79.

Staley, T.E. 2002. Low-level liming affects early white clover nodulations but not root development in a small-volume acidic soil model system. *Soil Science.* 167: 211-221.

Staley, T.E. 2003. Initial white clover nodulation under saturation levels of rhizobia relative to low-level liming of an acidic soil. *Soil Science.* 168: 540-551.

Steel, R.G.D., and Torrie, J.H. 1980. Principles and procedures of statistics: A Biometrical Approach. 2nd Edition, McGraw-Hill Book Co., New York, NY. 633p.

Stokkermans, T.J.W., and Peters, N.K. 1994. *Bradyrhizobium elkanii* lipo-oligosaccharide signals induce complete nodule structures on *Glycine soja* Siebold et Zucc. *Planta.* 193: 413-420.

Stokkermans, T.J.W., Ikeshita, S., Cohn, J., Carlson, R.W., Stacey, G., Ogawa, T., and Peters, N.K. 1995. Structural requirements of synthetic and natural product lipo-chitin oligosaccharides for induction of nodule primordia on *Glycine soja*. *Plant Physiology.* 108: 1587-1595.

Stougard, J. 2000. Regulators and regulation of legume root nodule development. *Plant Physiology*. 124: 531-540.

Supanjani, S., Habib, A., Donnelly, D., and Smith, D.L. 2003. Nod factor enhances calcium uptake in soybean. *Plant Physiology*. (submitted).

Sutherland, T.D., Bassam, B.J., Schuller, L.J., and Gresshoff, P.M. 1990. Early nodulation signals of the wild type and symbiotic mutants of soybean (*Glycine max*). *Molecular Plant-Microbe Interactions*. 3: 122-128.

Systma, K.J., Morawetz, J., Pires, J.C. Nepokroeff, M., Conti, E., Zjhra, M., Hall, J.C., and Chase, M.W. 2002. Urticalean rosids: circumscription, rosid ancestry, and phylogenetics based on *rbcL*, *trnL-F*, and *ndhF* sequences. *American Journal of Botany*. 89: 1531-1546.

Takamatsu, S., Shin, H-D., Paksiri, U., Limkaisang, S., Taguchi, Y., Binh, N.T., and Sato, Y. 2002. Two *Erysiphe* species associated with recent outbreak of soybean powdery mildew: results of molecular phylogenetic analysis based on nuclear rDNA sequences. *Mycoscience*. 43: 333-341.

Theberge, M.C., Prevost, D., and Chalifour, F.P. 1996. The effect of different temperatures on the fatty acid composition of *Rhizobium leguminosarum* bv. *viciae* in the faba bean symbiosis. *New Phytologist*. 134: 657-664.

Triplett, E.W., Breil, B.T., and Splitter, G.A. 1994. Expression of *tfx* and sensitivity to the rhizobial antipeptide trifolitoxin in a taxonomically distinct group of α-proteobacteria including the animal pathogen *Brucella abortus*. *Applied and Environmental Micribiology*. 60: 4163-4166.

Truchet, G., Barker, D.G., Camut, S., Billy, F., Vasse, J., and Huguet, T. 1989. Alfalfa nodulation in the absence of *Rhizobium*. *Molecular and General Genetics*. 219: 65-68.

Truchet, G., Debelle, F., Vasse, J., Terzaghi, B., Garnerone, A. M., Rosenberg, C., Batut, J., Maillet, F., and Denarie, J. 1985. Identification of a *Rhizobium meliloti* pSym2011 region controlling the host specificity of root hair curling and nodulation. *Journal of Bacteriology*. 164: 1200-1210.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Prome, J-C., and Denarie, J. 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule. *Nature*. 351: 670-673.

Tu, J.C. 1978. Protection of soybean from severe *Phytophthora* root rot by *Rhizobium*. *Physiological Plant Pathology*. 12: 233-240.

Tu, J.C. 1979. Evidence of differential tolerance among some root rot fungi to rhizobial parasitism *in vitro*. *Physiological Plant Pathology*. 14: 171-177.

Tu, J.C. 1981. Effect of salinity on rhizobium-root-hair interaction, nodulation and growth of soybean. *Canadian Journal of Plant Science*. 61: 231-239.

van Brussel, A.A.N., Bakhuizen, R., van Spronsen, P.C., Spaink, H.P., Tak, T., Lugtenberg, B.J.J., and Kijne, J.W. 1992. Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of *Rhizobium*. *Science*. 257: 70-72.

Vanworkum, W.A.T., Vanbrussel, A.A.N., Tak, T., Wijffelman, C.A., and Kijne, J.W. 1995. Ethylene prevents nodulation of *Vicia sativa* spp *nigra* by exopolysaccharide-deficient mutants of *Rhizobium leguminosarum* bv *Viciae*. *Molecular Plant-Microbe Interactions*. 8: 278-285.

Verma, D.P.S. 1992. Signals in root nodule organogenesis and endocytosis of *Rhizobium*. *The Plant Cell.* 4: 373-382.

Vincent, J.M. 1970. A Manual for the Practical Study of Root Nodule Bacteria. Blackwell Scientific Publication, Oxford. 164p.

Wais, R.J., Keating, D.H., Long, S.R. 2002. Structure-function analysis of Nod factor-induced root hair calcium spiking in *Rhizobium*-legume symbiosis. *Plant Physiology.* 129: 211-224.

Walsh, S.P., and Layzell, D.B. 1986. Carbon and nitrogen assimilation and partitioning in soybeans exposed to low root temperatures. *Plant Physiology.* 80: 249-255.

Walters, D., Cowley, T., and Mitchell, A. 2002. Methyl jasmonate alters polyamine metabolism and induces systemic protection against powdery mildew infection in barley seedlings. *Journal of Experimental Botany.* 53: 747-756.

Wang, S.P., and Stacey, G. 1990. Ammonia regulation of nod genes in *Bradyrhizobium japonicum*. *Molecular and General Genetics.* 223: 329-331.

Waterer, J.G., and Vessey, J.K. 1993. Effect of low static nitrate concentrations on mineral nitrogen uptake, nodulation, and nitrogen fixation in field pea. *Journal of Plant Nutrition.* 16: 1775-1789.

Wennemann, L. 2002. Crop protection and the agenda 21. *Gesunde Pflanzen.* 54: 16-22.

Whigham, D.K., and Minor, H.C. 1978. Agronomic characteristics and environmental stress. In G.A. Norman, ed., *Soybean Physiology, Agronomy, and Utilization,* pp. 77-118. Academic Press, New York, NY.

Wrather, J.A., Anderson, T.R., Arsyad, D.M., Tan, Y., Ploper L.D., Porta-Puglia, A., Ram, H.H., and Yorinori, J.T. 2001. Soybean disease loss estimates for the top ten soybean-producing countries in 1998. *Canadian Journal of Plant Pathology.* 23: 115-121.

Xie, Z-P., Müller, J., Wiemken, A., Broughton, W.J., and Boller, T. 1997. Nod factors and tri-iodobenzoic acid stimulate mycorrhizal colonization and affect carbohydrate partitioning in mycorrhizal roots of *Lablab purpureus. New Phytologist.* 139: 361-366.

Xie, Z-P., Staehelin, C., Vierheilig, H., Wiemken, A., Jabbouri, S., Broughton, W.J., Vögeli-Lange, R., and Boller, T. 1995. Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans. *Plant Physiology.* 108: 1519-1525.

YaJun, L., ShengQi, C., KeQin, Z., and ZhiYang, D. 2001. Studies on inductive action of chitin oligosaccharides for wheat resistance to *Blumeria graminis. Plant Protection.* 27: 1-3.

Zhang, F., Máce, F., and Smith, D.L. 2000. Mineral nitrogen availability and isoflavonoid accumulation in the root systems of soybean (*Glycine max* (L.) Merr.). *Journal of Agronomy and Crop Science-Zeitschrift fur Acker und Pflanzenbau.* 184:197-204.

Zhang, F., and Smith, D.L. 1994. Effects of low root zone temperature on the early stages of symbiosis establishment between soybean (*Glycine max. (L.)* Merr) and *Bradyrhizobium japonicum*. *Journal of Experimental Botany.* 279: 1467-1473.

Zhang, F., and Smith, D.L. 1995. Preincubation of *Bradyrhizobium japonicum* with genistein accelerates nodule development of soybean at suboptimal root zone temperatures. *Plant Physiology.* 108: 961-968.

Zhang, F., and Smith, D.L. 1996. Genistein accumulation in soybean (*Glycine max* [L.] Merr.) root systems under suboptimal root zone temperatures. *Journal of Experimental Botany.* 47: 785-792.

Zhang, F., Charles, T.C., Pan, B., and Smith, D.L. 1996. Inhibition of the expression of *Bradyrhizobium japonicum* nod genes at low temperatures. *Soil Biology and Biochemistry.* 28: 1579-1583.

Zhang, F., Lynch, D. H., and Smith, D. L. 1995. Impact of low root temperatures in soybean [*Glycine Max.* (L.) Merr] on nodulation and nitrogen fixation. *Environmental and Experimental Botany.* 35: 279-285.

Zhang, H., Prithiviraj, B., Souleimanov, A., D'Aoust, F., Charles, T.C., Driscoll, B.T., and Smith, D.L. 2002. The effect of temperature and genistein concentration on lipo-chitooligosaccharide (LCO) production by wild-type and mutant strains of *Bradyrhizobium japonicum*. *Soil Biology and Biochemistry*. 34: 1175-1180.