THE EFFECT OF THURICIN 17 AND LIPO-CHITOOLIGOSACCHARIDE ON SOYBEAN AND POTATO GROWTH AND DEVELOPMENT

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ABSTRACT

The second half of the 20th century saw widespread deployment of green-revolution agricultural technologies, some of which eventually proved to be environmentally problematic. To further improve crop yields more subtle interventions are now needed. Thuricin 17 (Th17) is a small protein produced by a rhizobacterium; it improves crop growth, particularly under stressful conditions. There is still much to learn regarding the commercial potential of Th17. Lipo-chitooligosaccharides (LCOs) cause plant growth promotion activity similar to Th17 and are now in the market place, however, Th17 has some clear advantages over LCOs (generally produces greater responses, faster and cheaper to produce, effective at lower concentrations). Thus, Th17 was compared with LCO application, in an effort to understand the potential of Th17 in crop production systems. A mix of controlled environment and field experiments were conducted on soybean and potato, and provided a clearer indication of the applied potential of Th17. Germination assays were conducted on soybean under growth chamber conditions, while both greenhouse and field trials were conducted on soybean and potato. Soybean germination assays conducted under both moderately- and severely-stressful low temperature conditions confirmed that the early growth enhancement response of both Th17 and LCO are greater under severely-low than moderately-low temperature stress conditions. Data were collected on growth and development variables of soybean; such as height, number of tri-foliate leaves, leaf area, dry weight of shoots, and number and dry weight of nodules. Besides this, data on number of pods and seeds per plant were also collected to determine effects on yield components. Greenhouse experiments showed that LCO treatment increased root biomass and Th17 treatment increased nodule weight. For potato, data were collected on yield (total number and for specific grades of tubers, including below market size, market size and above market size tubers). Both Th17 and LCO treatments had positive effects on the proportional increase in total and marketable yield under greenhouse and field condition. Collectively, these studies showed the potential to expand our understanding of the relatively recent and unexpected finding that Th17 and LCO effects interact with crop stress levels, and to

apply this finding to allow increased production of soybean, potato, and eventually a wider range of crops, through the use of novel low-input technologies.

RÉSUMÉ

La seconde moitié du 20^{e} siècle a été le siège d'un large déploiement de techniques issues de la révolution verte, à la longue certaines se sont révélées être problématiques pour l'environnement. Des changements plus subtils sont maintenant nécessaires pour continuer d'améliorer le rendement des cultures. La thuricine 17 (Th17) est une petite protéine produite par une rhizobactérie; elle améliore la croissance des cultures, particulièrement en conditions de stress. Il reste beaucoup à apprendre sur le potentiel commercial de la Th17. Les lipo-chitooligosaccharides (LCO) promeuvent la croissance des plantes d'une manière similaire à la Th17 et sont en vente sur le marché, par contre, la Th17 possède des avantages clairs en comparaison aux LCO (généralement plus efficace et à des concentrations plus faibles, plus rapide et moins coûteuse à produire). Donc, l'efficacité de la Th17 et des LCO ont été comparées, pour comprendre le potentiel de la Th17 en production agricole. Un mélange d'expériences en environnement contrôlé et en champ ont été réalisées sur la fève soya et la pomme de terre, et ont clarifiés le potentiel appliqué de la Th17. Des essais de germination ont été réalisés sur des fèves soya en chambre de culture, et des essais en serre et en champs ont été réalisés sur des fèves soya et des pommes de terre. Les essais de germination réalisés sous conditions de stress modéré et sévère de froid ont confirmé que l'amélioration du début de la croissance par la Th17 et les LCO est plus élevée sous stress sévère que modéré. Des données ont été récoltées sur des variables de croissance et de développement du soya telles que; la hauteur, le nombre de feuilles trifoliées, l'aire foliaire, le poids sec des pousses et le nombre et poids sec des nodules. De plus, des données sur le nombre de cosses et fèves ont été récoltées pour déterminer l'effet sur le rendement. Les expériences en serre ont montré que le traitement aux LCO augmente la biomasse des racines et que la Th17 augmente le poids des nodules. Pour la pomme de terre, des données de rendement ont été récoltées (nombre total de tubercules, de poids commercial mais aussi celles en dessous ou le dépassant). Les traitements de Th17 et LCO ont eu des effets positifs sur le rendement total de tubercules et sur celles de poids commercial, et ce, en serre comme en champs. Ensemble, ces études montrent le potentiel d'étendre nos connaissances au sujet de la découverte récente et surprenante que les effets de la Th17 et des LCO interagissent

avec les niveaux de stress des cultures, et d'appliquer cette découverte pour permettre l'augmentation du rendement du soya, de la pomme de terre et plus tard d'une gamme plus large de cultures, via l'utilisation de technologies nouvelles à faible apport.

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Contributions of Authors to Manuscripts

This thesis is written in manuscript format and follows the guidelines for "Thesis preparation and submission" suggested by Graduate and Post-doctoral Studies of McGill University. It consists of two manuscripts: Chapters 3 and 4.

The co-authors for these two manuscripts include: Dr. Donald Smith, Research Associate Dr. Alfred Souleimanov, Research Associate Dr. Inna Teshler and Ms. France Rochette. Dr. Donald Smith, my supervisor, helped me with his valuable suggestions from the start to end of the research. He also went through my manuscripts several times to review and edit the written documents. Research Associate Dr. Alfred Souleimanov helped me in extraction and purification of Th17 and LCO. Research Associate Dr. Inna Teshler helped me conduct the greenhouse experiment and the field experiments. Ms. France Rochette, from DuPont Canada, provided valuable suggestions while I was running my experiments, provided data from the potato field trial in Ontario and acted as liaison between DuPont/Pioneer and my laboratory at McGill.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CFS	Cell-free Supernatant
Cfu	Colony Forming Cells
CRD	Completely Randomized Design
ePGPR	Extracellular Plant Growth Promoting Rhizobacteria
FAB-MS	Fast-atom Bombardment Mass Spectrometry
GLM	General Linear Model
HPLC	High-performance Liquid Chromatography
iPGPR	Intracellular Plant Growth Promoting Rhizobacteria
ISR	Induced Systemic Resistance
LCOs	Lipo-chitooligosaccharides
OD	Optical Density
PGPR	Plant Growth Promoting Rhizobacteria
RCBD	Randomized Complete Block Design
RZT	Root-zone Temperature
SAS	Statistical Analysis System
Th17	Thuricin 17
VOCs	Volatile Organic Compounds
YEM	Yeast Extract Mannitol

Chapter 1 GENERAL INTRODUCTION

1.1 Introduction

Plant growth promoting rhizobacteria (PGPR), a term first use by Kloepper and coworkers (Kloepper and Schroth, 1978), are the free living bacteria which exist in the rhizosphere and have beneficial importance in agriculture. The rhizosphere is the very thin area around the plant roots, which is very rich in plant exudates and micro-organisms, as opposed to bulk soil further from the roots. PGPR are found in the rhizosphere, either in the soil near plant roots, on the surface of plant roots or inside the cells of root nodules (Gray and Smith, 2005) and are able to stimulate plant growth through a wide array of mechanisms. Host plants and PGPR maintain a symbiotic relationship: PGPR enhance the growth of the host plant and the micro-organism utilises the root-exudates of the host plant. These exudates contain various organic compounds, such as amino acids, fatty acids and other organic acids, nucleotides, plant growth regulators, sugars and vitamins (Uren, 2007), thereby changing the physical and chemical properties of soil in rhizosphere (Griffiths *et al.*, 1999; Xu, 2000) making it more suitable for the micro-organisms, in comparison to the bulk soil outside the rhizosphere.

In the broadest sense PGPR include the legume-nodulating rhizobia, and as such can be separated into those that reside outside plant cells (extracellular – ePGPR) and the rhizobia that reside, in the context of symbiosis, inside plant cells (intracellular – iPGPR) (Gray and Smith, 2005) and stimulate plant growth either directly or indirectly (Glick, 1995; Ortíz-Castro *et al.*, 2009). Direct modes of action include biological nitrogen fixation, increased availability of soil nutrients to plant roots, production of phytohormones, production of siderophores and induced systemic resistance, while indirectly they can suppress disease through antibiosis thereby lessening the deleterious effect of phyto-pathogens (Glick, 1995). Some PGPR can exploit more than one mechanism to enhance plant growth (Ahmad *et al.*, 2008).

PGPR have been regularly exploited to enhance the emergence, growth and overall yield of agricultural crop production systems. There are various examples of PGPR stimualting plant growth and development: by increasing leaf area, chlorophyll content

and total biomass of shoots or roots (Dobbelaere *et al.*, 2001; Esitken *et al.*, 2003; Mia *et al.*, 2010); enhancing seed germination, flowering and fruit set by production of gibberellins (Bottini *et al.*, 2004); increase in growth and yield of crops such as apple (Karlidag *et al.*, 2007); by inducing disease resistance in various crops (Liu *et al.*, 1995; Zehnder *et al.*, 2001; Ryu *et al.*, 2004).

The legume-rhizobia interaction is one of best studied and widely researched plant-microbe interactions. Rhizobia can form either symbiotic or non-symbiotic associations with plant roots. Rhizobial symbiotic interactions between plants and micro-organisms are facilitated by signal molecules (Walker *et al.*, 2003), which are detected by the interacting partners (Mabood and Smith, 2005). As a result of this interaction, special structures, nodules, are formed on plant roots. Nodulation in legumes is a complex process consisting of various steps, in a well-defined sequence. In the initial stage of nodulation phenolic compounds, flavanoids – plant-originating signals, are produced and trigger the activation of *nod* genes in rhizobia (Zaat *et al.*, 1987). These *nod* genes cause the production of nod factors, which are lipo-chitooligosaccharides (LCOs) – bacteria-originating signals (Schultze and Kondorosi, 1998). In general, most LCOs have similar structures, consisting of a 3-5 chitin unit backbone (a linear chain of β-1, 4- linked N-acetylglucosamines) linked to an acyl chain (Mergaert *et al.*, 1997), and with various modifications to the reducing and non-reducing ends of the chitin backbone. The LCOs produced by *Bradyrhizobium japonicum* (eg. Nod Bj-V C18:1, MeFuc) have a pentameric backbone with C18:1, C16:0 and C16:1 fatty acid chains at the non-reducing end and 2-0-methylfucose at the reducing end of the chitin backbone (Carlson *et al.*, 1993).

The PGPR *Bacillus thuringiensis* NEB17 was isolated from soybean nodules (Bai *et al.*, 2002) and was shown to increase growth and nodulation when applied as a co-inoculant with *B. japonicum* 532C (Bai *et al.*, 2003). This bacterium produces the bacteriocin Th17. Th17 has a molecular weight 3.1 kDa and is not toxic to *B. japonicum* 532C (Gray *et al.*, 2006). Bacteriocins are bacteria-produced peptides which generally kill bacteria that are closely related to the producer strain (Jack *et al.*, 1995), which provides a competitive advantage for the producer strain (Wilson *et al.*,

1998). It has been already demonstrated that the application of Th17, to either leaves or roots can enhance plant growth, early seedling growth, photosynthetic rate, soybean nodule number and total fixed N (Lee *et al.*, 2009). However, a great deal of research remains to be done regarding matters such as the range of crops affected, interactions with crop stress and their specific effects on crop physiology and development.

The initial findings with the LCOs have been widely repeated. Several LCO technologies from our lab are now commercially available. Novozymes is now marketing products based on these findings (http://bioag.novozymes.com/en/products/unitedstates/biofertility/Pages/default.aspx). Production of Th17 is substantially easier, and therefore more economic, than LCOs. The bacterium that produces Th17 (*B. thuringiensis* NEB 17) is fast growing (full cultural growth in less than 48 h) while *B. japonicum* is a slow growing bacterium (full culture in 7-10 days). *B. japonicum* produces small quantities of LCO, while a 0.5 L culture of *B. thuringiensis* NEB 17 can produce 0.5 g of Th17. Plant growth stimulation by Th17 is at the proof of concept stage; we do not fully understand the potential commercial application of Th17. The general objective of this study is to understand the potential for commercial application of Th17 as a crop growth enhancer, in comparison to LCO.

1.2 Hypotheses

1. For soybean, stimulation of germination caused by treatment with Th17 or LCO is greater as conditions become more stressful (low temperature stress in this case).
2. Growth stimulation of some crops is greater following treatment with Th17 than LCO.

1.3 Objectives

1.3.1 General objective: To understand the potential for commercial application of Th17 as a crop growth enhancer, in part by comparison with the already commercialized LCO**.**

1.3.2 Specific objectives:

1. Determine the role of stress responses in soybean growth enhancement by Th17, relative to LCO signals, through examination of responses under a moderately stressful temperature (22 ± 2 °C) and a more severely stressful temperature (stress conditions- 15 ± 2 °C).

2. Determine if both soybean and potato respond to the class IId bacteriocin signal Th17, and how this response, if it occurs, compares with the effects of LCO signals, under greenhouse conditions.

3. Determine the efficacy of thuricin 17, relative to LCO, in enhancing crop yield under field conditions for both soybean and potato.

Chapter 2: LITERATURE REVIEW

2.1 PLANT GRWOTH PROMOTING RHIZOBACTERIA

2.1.1 General background

Plant growth promoting rhizobacteria (PGPR), a term first used by Kloepper and coworkers (Kloepper and Schroth, 1978), are the free living bacteria which exist in the rhizosphere and have beneficial importance in agriculture. They can be found either in the soil near plant roots, on the surface of plant root systems, in spaces between root cells or inside specialized cells of root nodules. They are able to stimulate plant growth through a wide array of mechanisms. In the broad sense, PGPR can either be extracellular (ePGPR - found outside plant cells) or intracellular (iPGPR - found inside plant cells) (Gray and Smith, 2005). Our awareness of bacteria began in 1683 when Von Leeuwenhoek discovered them, but it seems almost certain that bacteria have existed in relationships with plant roots since plants colonized the land, almost half a billion years ago. There is evidence that they have been exploited, even in unwittingly, for a very long time. Practices like mixing soils for treating the defects in plant growth were suggested by Theophrastus 372-287 BC (Tisdale and Nelson, 1975), and growing legume crops in rotation in 30 BC (Chew, 2002), while commercial application of rhizobia inoculants has been practiced since the 1890s (Fred *et al.*, 1932). Because of their crop growth promoting activities, PGPR had been the subject of research since the mid-20th century, in the contexts of agronomy, plant breeding, and plant physiology.

PGPR and host plants maintain symbiotic relationships in which PGPR enhance plant growth and the micro-organism utilises the root-exudates of the host plant. These exudates contain various organic compounds, such as amino acids, fatty acids and other organic acids, nucleotides, plant growth regulators, sugars and vitamins (Uren, 2007). These exudates substantially change the physical and chemical properties of soil in rhizosphere (Griffiths *et al.*, 1999; Xu, 2000), in comparison to the surrounding bulk soil. This change in properties makes the rhizosphere more suitable to PGPR than other soil bacteria. However, the colonization of bacteria is not uniform; for instance, sometimes they are found in upper part of root and absent in root tips (Ma *et al.*, 2001).

2.1.2 Effect of PGPR on plant growth

PGPR can enhance the growth of plants either directly or indirectly. Directly, they help to increase the availability of plant nutrients, making them more available to plant roots or can synthesize growth promoting compounds. They can provide indirect benefit by removing or lessening the deleterious effect of phyto-pathogens (Glick, 1995). PGPR have been regularly exploited to enhance the emergence, growth and overall yield of agricultural crop production systems. With the use of PGPR, plant growth variables such as leaf area, chlorophyll content and total biomass of shoot or root are increased (Dobbelaere *et al.*, 2001; Esitken *et al.*, 2003; Mia *et al.*, 2010). These effects are seen not only in field crops; PGPR have been used to increase growth and disease resistance in some ornamental plants (Zehnder *et al.*, 2001), and have been shown to cause significant increases in tree growth and yield of apple (Karlidag *et al.*, 2007).

Besides growth and yield variables, PGPR can help to overcome loss of yield due to environmental stresses (Bowen and Rovira, 1999; Cook, 2000; Malhotra and Srivastava, 2009). Researchers found that use of PGPR can increase resistance to water stress in tomato and peppers (Mayak *et al.*, 2004), increase nodulation and nitrogen fixation in soybean at low root zone temperatures (Zhang *et al.*, 1996), and overcome stresses associated with flooded conditions (Grichko and Glick, 2001), heavy metal contamination in soil (Burd *et al.*, 1998), salt and drought (Egamberdieva, 2008; Zahir *et al.*, 2008; Kaymak *et al.*, 2009).

2.1.3 Mode of action for plant growth enhancement

The overall mode of action for plant growth enhancement by PGPR can either be direct or indirect (Ortíz-Castro *et al.*, 2009; Glick, 1995). Direct modes of action include biological nitrogen fixation, increased availability of soil nutrients to plant roots, production of phytohormones, production of siderophores and induced systemic resistance, while indirectly they can suppress disease through antibiosis. Some PGPR exploit more than one mechanism to enhance plant growth (Ahmad *et al.,* 2008). More recently, work in our laboratory has indicated that PGPR can produce specific signal compounds that lead to enhanced growth of plants (Pan *et al.*, 2002; Zhang *et*

al., 2003; Mabood *et al.*, 2006a; Mabood *et al.*, 2006b), particularly when they are stressed (unpublished data).

2.1.3.1 Fixation of atmospheric nitrogen

Some PGPR can fix atmospheric N_2, either symbiotically or non-symbiotically, and make it available to associated plants (Antoun *et al.*, 1998; Riggs *et al.*, 2001). Symbiotic N_2 fixation is carried out by bacteria such as the various rhizobia, and less rigidly symbiotic bacteria such as *Azotobacter* spp., *Bacillus* spp., *Beijerinckia* spp., etc., and free living diazotrophs that fix N_2 non-symbiotically include genera such as *Azoarcus*, *Azospirillum* and *Pseudomonas* (Reinhold-Hurek *et al.*, 1993; Bashan and de-Bashan, 2010). The latter may make N available in the rhizosphere.

2.1.3.2 Increasing the nutrient uptake

PGPR increase root surface area (German *et al.*, 2000; Holguin and Glick, 2001) thereby increasing the surface area for absorption. They can also increase the uptake of nutrients such as K, Fe, Cu, Mn and Zn through stimulation of proton pump ATPase activity (Mantelin and Touraine, 2004). Although large amounts of phosphorous may be present in soil, only a small proportion of this is available in forms plants can absorb (Stevenson and Cole, 1999). PGPR can change these largely insoluble forms of soil phosphorous into soluble forms (Glick, 1995; Hameeda *et al.*, 2008; Richardson *et al.*, 2009), which plants can absorb easily.

2.1.3.3 Production of phytohormones

PGPR can produce various phytohormones, such as IAA, cytokinins, gibberellins, and ethylene (Barazani and Friedman, 1999; Zakharova *et al.*, 1999; García de Salamone *et al.*, 2001; Somers *et al.*, 2004; Kloepper *et al.*, 2007; Babalola, 2010). IAA and cytokinins cause root initiation, cell division and cell enlargement (Salisbury, 1994). A range of PGPR has been reported to produce IAA, such as *Azospirillum brasilense* strain Sp 245 (Zakharova *et al.*, 1999) and *Acetobacter diazotrophicus* (Bastián *et al.*, 1998). PGPR extracted from the rhizosphere of

Brassica sp. have been shown to produce IAA and when used as seed inoculants increase seed yield and oil content (Asghar *et al.*, 2002).

Gibberellins, which enhance seed germination, flowering and fruit set, are also produced by some PGPR (Bottini *et al.*, 2004). Various species and strains of *Bacillus* are found to increase the growth in red peppers through this mechanism (Joo *et al.*, 2005). Besides these, some PGPR produce volatile organic compounds (VOCs) (Ryu *et al.*, 2003), for instance *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a, produce the volatile compounds 3-hydroxy-2-butanone and 2,3 butanediol, which enhance the growth of *Arabidopsis thaliana*. Another example is *Pseudomonas* sp. which produces HCN (Ahmad *et al.*, 2008).

2.1.3.4 Production of siderophores

Iron is an essential element for growth of plants and micro-organisms. Lack of iron creates competition for this resource amongst spatially associated organisms (Loper and Henkels, 1997). Some PGPR produce low molecular weight siderophores that can solubilise iron and form the Fe^{3+} siderophore complex (Carson *et al.*, 2000). *Ochrobactrum anthropi* TRS-2 produce siderophore *in vitro* and form this siderophore complex in association with tea plants (Chakraborty *et al.*, 2009). This complex is easily absorbed by the plants (Bar-Ness *et al.*, 1991; Wang *et al.*, 1993) but formation of this complex makes iron unavailable to pathogenic fungi (Loper and Henkels, 1999) and helps to control the growth of pathogens; for instance *Agrobacterium rhizogens* K84 produces an iron chelator that helps to control crown gall disease (Penyalver *et al.*, 2001).

2.1.3.5 Induced systemic resistance (ISR)

Use of specific PGPR, together or separately, helps to decrease the effect of, or induce resistance to, many pathogenic microorganisms (Ramamoorthy *et al.*, 2001; Kloepper *et al.*, 2004) through Induced Systemic Resistance (ISR). ISR was first observed in carnation, acting against wilt caused by *Fusarium* sp. (Van Peer *et al.*, 1991) and in cucumber against foliar disease, caused by *Colletotrichum orbiculare* (Wei *et al.*, 1991). Following these studies, various researchers have demonstrated

enhanced ISR activity through the use of various PGPR strains, in many crops, such as resistance against halo blight in bean stemming from treatment with *Pseudomonas fluorescens* (Alstrom, 1991); use of *Pseudomonas putida* strain 89B-27 and *Serratia marcescens* strain 90-166 against fusarium wilt in cucumber (Liu *et al.*, 1995); decreased incidence of yellow mosaic polyvirus (BYMV) through seed inoculation with *Pseudomonas florescens* and *Rhizobium leguminosarum* in faba bean (Elbadry *et al.*, 2006). In addition, various species of *Bacillus,* such as *B. amyloliqufaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. mycoides*, *and B. pumilus* can enhance disease resistance in a range of host species (Ryu *et al.*, 2004).

2.1.3.6 Antibiosis

PGPR can suppress disease through a range of actions; one of them is through antibiosis (Whipps, 2001; Jing *et al.*, 2007). For example *Bacillus* spp. produces phenazine antibiotics and enhances plant growth (Choudhary and Johri, 2009) through competition with pathogenic microorganisms. Various other antibiotics are produced by PGPR, such as HCN (Ahmad *et al.*, 2008), pyoluteorin (Baehler *et al.*, 2006) and auxofuran (Riedlinger *et al.*, 2006) produced by *Pseudomonas* species.

2.1.4 Challenges and future perspectives

PGPR have been the subject of agricultural research for quite a long time. It has been shown that PGPR enhance the growth, development and yield of crop plants. They show very promising results under controlled environmental growth conditions, but the increase in growth and yield has not been consistent under field conditions (Nelson, 2004). There are a wide range of biotic and abiotic factors present in the field (such as extremes of temperature, agronomic practices, drought, soil physical and chemical properties) that prevent these bacteria from providing the same beneficial results seen under controlled environment conditions, in the same way that low root zone temperatures inhibit signal exchange between soybean roots and the symbiotic bacterium *B. japonicum* (Zhang and Smith, 1994).

Developing research approaches and technologies focused on interactions between edaphic factors affecting the impact of PGPR on plant growth and disease

suppression, and best formulations for resulting technologies can help overcome these limitations in the field. For example, addition of an isoflavonoid, plant-to-bacteria signal (genistein) to *B. japonicum* inoculants accelerated nodulation and N_2 fixation, increasing soybean growth (Zhang and Smith, 1995). In addition, various biotechnology tools can be applied to improve the qualities of proven PGPR strains, for easy formulation and improved plant colonization; part of this might be accomplished through development of transgenic strains which have more than one mode of action (Chin-A-Woeng *et al.*, 2001; Huang *et al.*, 2004). Isolation of bacterial strains and their modification using a range of technologies can help us move toward sustainable agriculture, thereby minimizing the use and negative effects of chemical fertilizers on soil.

2.2 PLANT SIGNALS IN PLANT-MICROBE INTERACTIONS

The legume-rhizobia interaction is one of best studied and widely researched plant-microbe interactions. As mentioned above, the rhizosphere is a narrow region around the root that is comparatively rich in micro-organisms and plant root exudates, as compared to bulk soil. These micro-organisms can have either good or deleterious effects on the plant growth. Many such micro-organisms can form either symbiotic or non-symbiotic associations with plant roots. Such interactions between plants and micro-organisms are facilitated by signal molecules (Walker *et al.*, 2003), which are detected by the interacting partners (Mabood and Smith, 2005). As a result of this interaction, special structures, nodules, are formed on plant roots. Nodulation in legumes is a complex process consisting of several steps. In the initial stage of nodulation phenolic compounds, flavonoids – plant-originating signals, are produced and trigger the activation of *nod* genes in rhizobia (Zaat *et al.*, 1987). These *nod* genes initiate the production of nod factors, which are lipo-chitooligosaccharides (LCOs) – bacteria-originating signals (Schultze and Kondorosi, 1998).

2.2.1 Flavonoids and isoflavonoids

Flavonoids are the polyphenolic compounds which include subclasses such as: chalcones, flavones, isoflavones, flavonols, flavanones and isoflavonoids. These metabolites are synthesized through the phenyl-propanoid pathway, which is one of

the best studied biosynthetic pathways. These flavonoids can be synthesized by plants and released into the rhizosphere through root decomposition, root injury (Shaw *et al.*, 2006) or through active root exudation (Cesco *et al.*, 2010). These compounds can also attract rhizobia (Currier *et al.*, 1976), regulate root growth and development (Buer *et al.*, 2010; Rao 1990) and protect plants against pest organisms and diseases (Dakora and Phillips, 2002; Dakora, 2003). However, there are various factors which can affect the type and amount of flavonoid production by plants, such as plant species and cultivar; cultivation system; biotic stress; nutrient availability and plant development (Cesco *et al.*, 2010).

Flavonoids are the best known plant signals in the nodulation process. As part of this they influence plant defense, attract various rhizobia (Caetano-Anollés *et al.*, 1988) and increase the growth of many of these rhizobial bacteria (Hartwig and Phillips, 1991). The presence of flavonoids in the rhizosphere can also increase the further release of flavonoids from plant roots (Recourt *et al.*, 1991; Dakora *et al.*, 1993). It is well known that in these symbiotic associations of bacteria and plant roots, the bacteria fix the atmospheric nitrogen into the form plants can use to meet their nitrogen requirements; plants, in return, provide the reduced carbon to the bacteria. Before this symbiotic association is fully established, flavonoids act as regulators to initiate *nod* gene activity in rhizobia (Dakora and Phillips, 1996). These *nod* genes synthesize Nod factors specific to rhizobial species and strains. Without these Nod factors the bacteria are not able to enter the plant root and form nodules (Relić *et al.*, 1994).

2.2.2 Lipo-chitooligosaccharides (LCO)

Nod factors are LCOs, bacteria-to-plant signals, which are produced as the result of plant-microbe interactions. Specific rhizobial species and subspecies produce specific suites of Nod factors. These Nod factors initiate nodulation and facilitate the entry of rhizobia into the plant root system (Relić *et al.*, 1994; Spaink, 1996). In general, most LCOs have similar structures, consisting of a 3-5 chitin unit backbone (a linear chain of β-1, 4- linked N-acetylglucosamines) linked to an acyl chain (Mergaert *et al.*, 1997), and with various modifications to the reducing and non-reducing ends of

the chitin backbone. The LCOs produced by *Bradyrhizobium japonicum* (eg. Nod Bj-V $C_{18:1}$, MeFuc) have a pentameric backbone with $C_{18:1}$, $C_{16:0}$ and $C_{16:1}$ fatty acid chains at the non-reducing end and 2-0-methylfucose at reducing end, of the chitin backbone (Carlson *et al.*, 1993).

The initial discovery that the LCOs produced by rhizobia, to signal legume symbiotic partners, are able to stimulate plant growth directly (Souleimanov *et al.*, 2002; Prithiviraj *et al.*, 2003; Jose Almaraz *et al.*, 2007) has been confirmed by others; Oláh *et al.* (2005) confirmed LCO stimulation of root growth in *Medicago truncatula*; Chen *et al.* (2007) showed that LCO spray on tomato accelerates flowering (a typical response to stress), and increases yield. LCO like molecules also stimulate early somatic embryo development in Norway spruce (Dyachok *et al.*, 2002). Enhanced germination and seedling growth, along with the mitogenic nature of LCOs, suggest accelerated meristem activity. This may lead to increased sink demand and the observed increases in mobilization of seed reserves (Prithiviraj *et al.*, 2003) and increased photosynthetic rates (Jose Almaraz *et al.*, 2007) for more developed plants; both of which lead to increased growth (Khan *et al.*, 2008). Several compounds secreted by other rhizobacteria cause similar effects (Lee *et al.*, 2009), although chemically they are quite different (proteins) (Gray *et al.*, 2006a; Gray *et al.*, 2006b) from the chitin based LCOs.

In the 1990s, it was discovered that low root zone temperatures inhibit signal exchange between soybean roots and the symbiotic bacterium *B. japonicum* (Zhang and Smith, 1994; Zhang *et al.*, 1996; Zhang and Smith, 1996); addition of an isoflavonoid plant-to-bacteria signal (genistein) (Stacey *et al.*, 1995; Dakora and Phillips, 1996) to *B. japonicum* inoculants accelerated nodulation and N_2 fixation, increasing soybean growth (Zhang and Smith, 1995) and yield (Zhang and Smith, 1996). Other researchers have confirmed and extended these findings (Begum *et al.*, 2001a, b; Macchiavelli and Brelles-Marino, 2004).

During field experiments with genistein treated rhizobia, it was observed that this treatment accelerated seedling emergence. This was surprising as emergence is prior to completion of nodulation and the onset of nitrogen fixation. Growth chamber

experiments using soybean seeds treated with genistein, *B. japonicum* cells or *B. japonicum* cells treated with genistein (and therefore producing LCOs – Stacey *et al.*, 1995) showed that only genistein treated *B. japonicum* cells accelerated seedling emergence, suggesting that the growth stimulation was due to LCOs. LCOs were isolated from *B. japonicum* and it was confirmed that they accelerate seed germination, seedling emergence, root growth and development in soybean and also have this effect in non-leguminous plants (Souleimanov *et al.*, 2002; Prithiviraj *et al.*, 2003).

When applied to soybean leaves, LCO from *B. japonicum* caused transient (2-5 day) increases in photosynthetic rate under growth chamber conditions and increased soybean yield (~25%) under field conditions (Jose Almaraz *et al.*, 2007). While the increase in photosynthetic rate due to the application of bacteria-to-plant signal molecules is new, previous studies have suggested that symbiotic microbes increase plant photosynthetic rates: rhizobia (Maury *et al.* 1993), mycorrhizal fungi (Kucey and Paul, 1982), plant growth promoting rhizobacteria (Zhang *et al.*, 1996; Bai *et al.*, 2003) and bacteria that produce lumichrome (Phillips *et al.*, 1999). It is often assumed that these increases in photosynthetic rates are related to increased sink demand by the symbionts. Increased sink demand causes increased photosynthetic rates, while reduced sink strength has the opposite effect (Pieters *et al.*, 2001; Tekalign and Hammes, 2005). Cockcroft *et al.* (2000) showed that tobacco plants engineered to have a faster cell cycle grow substantially faster; presumably because of increased sink strength in more active meristems, leading to increased photosynthetic rates. LCOs are known to activate specific plant genes and act as mitogens (Long, 1989; Dénarié *et al.*, 1996; Perret *et al.*, 2000; Patriarca *et al.*, 2004).

Foliar application of LCOs induces resistance of soybean plants to powdery mildew (Duzan *et al.*, 2005), associated with an increase in phenylalanine ammonia lyase (PAL), the first committed step of the phenylpropanoid pathway, leading to biosynthesis of antimicrobial compounds such as phytoalexins and salicylic acid. Given that LCOs induce defence responses in *Medicago* cell cultures and roots (Savouré *et al.*, 1997), that LCOs show structural similarity to chitin (they have a chitin backbone), and that chitin induces defence responses in plants (Khan *et al.*,

2003), it is reasonable to hypothesize that LCOs induce aspects of plant defence responses, similar to chitin.

In legume-rhizobia interactions, LysM-domain containing receptor-like kinases (LysM RLKs) are responsible for the binding of nod factors (Limpens *et al.*, 2003; Madsen *et al.*, 2003; Radutoiu *et al.*, 2003). These LysM domains have direct binding affinities for N-acetylglucosamine oligomers and the binding affinity increases with the number of chitin repeats (Ohnuma *et al.*, 2008). Broghammer *et al*. (2012) showed that in root cells of *Lotus japonicus*, Nod factor receptor 1 (NFR1) and Nod factor receptor 2 (NFR2) help the nod factors bind to the high affinity site.

2.2.3 Thuricin17

The PGPR *B. thuringiensis* NEB17 was originally isolated from soybean nodules (Bai *et al.*, 2002) and enhances nodulation when applied as a co-inoculant with *B. japonicum* 532C (Bai *et al.*, 2003). More recently, it had been shown that this bacterium produces a novel antimicrobial peptide (bacteriocin), now named thuricin-17, molecular weight 3.1 kDa (Gray *et al.*, 2006b). This bacteriocin is not toxic to *B. japonicum* 532C (Gray *et al.*, 2006b). Gray *et al*. (2006b) also noted the existence of a similar bacteriocin (bacthurcin F4), and based on these two molecules proposed a new class of bacteriocins: class IId.

Bacteriocins are bacteria-produced peptides that are either bacteri-cidal or -static to specific bacterial strains, often those closely related to the producer strain (Jack *et al.*, 1995), strains that would compete most closely with the producer strains. They provide competitive advantage for the producer strain (Wilson *et al.*, 1998) and may enhance nodule occupancy when the producer strain is one of the rhizobia (Oresnik *et al.*, 1999). Bacteriocins are often isolated from bacteria found in food, such as strains of *Bacillus* (Oscáriz *et al.*, 1999). However, some bacteriocins have been isolated from ePGPR, such as *Pseudomonas* spp. (reviewed in Parret and De Mot, 2002) and the nodulating *iPGPR*, such as *Rhizobium leguminosarum* 248 (Oresnik *et al.*, 1999). It is presumed that bacteriocins produced by PGPR provide a competitive advantage to the producer strains (Wilson *et al*., 1998).

Treatment with class IId bacteriocins can stimulate the growth of corn and soybean (Lee *et al.*, 2009) under laboratory conditions. This growth stimulation is similar in nature to that caused by LCOs. A protein produced by a *Serratia* PGPR strain also increases photosynthesis and growth (Bai *et al.* 2002).

The initial findings with LCOs have been widely repeated and publications on this have begun to appear (e.g. Olah *et al.* 2005; Chen *et al.* 2007); Novozymes Crop Biosciences is now marketing products based on these findings (http://bioag.novozymes.com/en/products/unitedstates/biofertility/Pages/default.aspx), as are Becker Underwood (http://www.beckerunderwood.com/news/becker-underwood-granted-exclusive-rights-for-patented-nitrogen-fixing-technology/). More recently, researchers at DuPont Canada Crop Protection and Pioneer Canada have confirmed the stimulation of plant growth by Th17 (unpublished data). Production of Th17 is substantially easier, and therefore more economic, than LCOs. The bacterium that produces Th17 (*B. thuringiensis* NEB 17) is fast growing (full cultural growth in less than 48 h) while *B. japonicum* is a slow growing bacterium (full culture in 7-10 days). *B. japonicum* produces small quantities of LCO, while a 2 L culture of *B. thuringiensis* NEB 17 can produce 0.5 g of Th17. Plant growth stimulation by Th17 is at the proof of concept stage; we do not fully understand the potential commercial application of the recently discovered class IId bacteriocin protein signals.

Connecting statement for Chapter 3

This manuscript was prepared by me, Dr. Donald Smith, Dr. Alfred Souleimanov, Dr. Inna Teshler and Ms. France Rochette. The references cited are presented at the end of the thesis while the tables and figures are presented at the end of Chapter 3.

This manuscript describes how Th17 and LCO treatments can enhance the growth and development of soybean plants under various growing conditions, ranging from germination chamber to the field. The results from this section make clear that Th17 and LCO can enhance the germination of soybean seeds and also enhance various growth variables, such as leaf area, dry biomass of roots, number of nodules and dry biomass of nodules. Collectively, these studies showed that Th17 has potential to be used as a commercial technology, as a crop growth enhancer for soybean.

For this manuscript, I was responsible for compiling the literature reviews; setting up the all the germination, greenhouse and field experiments; collection of the samples from all studies; processing of samples and data analysis.

Of the co-authors: my supervisor Dr. Donald Smith helped me with his valuable suggestions from the start to the end of the research. He also went through my manuscripts several times to review and edit the text and other elements. Dr. Alfred Souleimanov helped with extraction and purification of LCO and Th17, which were used in my treatments. Dr. Inna Teshler helped me in establishing the greenhouse and field experiments. Ms. France Rochette, of DuPont Canada, provided valuable suggestions during my studies and the thesis writing period.

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Chapter 3

THE EFFECTS OF THURICIN17 (TH17) AND LIPO-CHITOOLIGOSACCHARIDES (LCO) ON SOYBEAN INTERACT WTH STRESS LEVEL

3.1 Abstract

Many technologies have been developed to increase the productivity of crops, but together with the increased production a large number of these technologies have also led to environmentally problematic conditions. As our understanding of plants improves we are being presented with more subtle interventions able to improve crop yields. Thuricin 17 (Th17) is a small protein with bacteriocin activity; it has also been shown to improve the growth of crop plants. Lipo-chitooligosaccharides (LCOs), which are also produced by rhizobacteria, have plant growth promoting properties similar to Th17 and are now used in commercial crop cultivation. Thus, Th17 was compared with LCO application, in an effort to understand the potential of Th17 for the full development leading to its deployment in the market place. Experiments were conducted on soybean to determine the effectiveness of Th17 relative to LCO under various environmental conditions in growth chambers, a greenhouse and in the field. Germination assays were conducted in growth chambers at both mildly stressfull growing conditions and under severely low temperature stress conditions. This study confirmed that the early growth response of both Th17 and LCO are greater under more stressful conditions, in this case low temperature stress. Various growth and development variables were collected from greenhouse and field experiments. A greenhouse study showed that both Th17 and LCO increased the dry weight of pods and roots. Th17 treatment resulted in the smallest number of nodules but the highest dry weight of nodules. The study of Th17 has the potential to expand our understanding of a relatively recent and unexpected finding; and to understand how best apply this finding to allow increased production of a wide range of crops. Collectively, these results indicate that Th17 has potential in this regard.

3.2 Introduction

Plant growth promoting rhizobacteria (PGPR), a term first use by Kloepper and coworkers (Kloepper and Schroth, 1978), are the free living bacteria which exist in the rhizosphere and have beneficial importance in agriculture. They can be found either in the soil near plant roots, on the surface of plant roots or inside the cells of root nodules (Gray and Smith, 2005) and are able to stimulate plants growth through a wide array of mechanisms. The more widely recognized mechanisms through which PGPR increase

plant growth include: 1. Production of phytohormones, 2. Production of metal chelating siderophores, 3. Induction of induced systemic resistance (ISR) and 4. Suppression of disease through antibiosis (Whipps, 2001). In the broadest sense PGPR include the legume-nodulating rhizobia, and as such can be separated into those that reside, outside plant cells (extracellular – ePGPR) and the rhizobia that reside, in the context of symbiosis, inside plant cells (intracellular – iPGPR) (Gray and Smith, 2005). Application of PGPR to crop production systems, with the exception of rhizobia, has met with mixed results. PGPR are often quite unreliable in the field, causing increases crop growth sometimes and not others (Nelson, 2004), suggesting that there is much we do not understand about them.

The nodulation of legumes, during establishment of the N_2 fixing symbiosis, is a multi-step process. Initially, phenolic compounds, flavonoids, are produced which trigger activity of *nod* genes in rhizobia. These *nod* genes initiate the production of nod factors, which are LCOs (Schultze and Kondorosi, 1998). In general all nod factor LCOs have similar structures, consisting of a 3-5 chitin unit backbone (a linear chain of β-1, 4- linked N-acetylglucosamines) linked to an acyl side chain (Mergaert *et al.*, 1997). The initial discovery that LCOs produced by rhizobia, such as *B. japonicum*, to signal legume symbiotic partners, are able to stimulate plant growth directly (Souleimanov *et al.*, 2002; Prithiviraj *et al.*, 2003; Jose Almaraz *et al.*, 2007) has now been confirmed by others; Oláh *et al.* (2005) confirmed LCO stimulation of root growth in *Medicago truncatula*; Chen *et al.* (2007) showed that LCO spray on tomato accelerates flowering (a typical response to stress), and increases yield. Enhanced germination and seedling growth, along with the mitogenic nature of LCOs (Spaink *et al.*, 1991; Truchet *et al.*, 1991), suggest accelerated meristem activity (Prithiviraj *et al.*, 2003). Several compounds secreted by other rhizobacteria cause similar effects (Lee *et al.*, 2009), although chemically they are quite different (proteins - Gray *et al.*, 2006a; Gray *et al.*, 2006b) from the chitin based LCOs.

The PGPR *Bacillus thuringiensis* NEB17 was isolated from soybean nodules (Bai *et al.*, 2002) and was shown to increase growth and nodulation when applied as a co-inoculant with *B. japonicum* 532C (Bai *et al.*, 2003). This bacterium produces the

bacteriocin Th17, with molecular weight 3.1 kDa, which is not toxic to *B. japonicum* 532C (Gray *et al.*, 2006b). Bacteriocins are bacteria-produced peptides which generally kill bacteria that are closely related to the producer strain (Jack *et al.*, 1995), which provides a competitive advantage for the producer strain (Wilson *et al.*, 1998). It has been already demonstrated that the application of Th17, to either leaves or roots, can enhance plant growth. Use of this bacteriocin can enhance early seedling growth, photosynthetic rate, soybean nodule number and total fixed N (Lee *et al.*, 2009). However, a great deal of research remains to be done regarding matters such as the range of crops affected, interaction with crop stress and their specific effects on crop physiology and development.

The general objective of this study is to understand the potential for commercial application of Th17 as a crop growth enhancer. Three studies were conducted under different growing conditions, to determine the responses of soybean to Th17, in comparison to a water only negative control and LCO as a positive control.

3.3 Materials and methods

3.3.1 Extraction of Lipo-chitooligosaccharides (LCO)

B. japonicum strain 532C was cultured in Yeast Extract Mannitol (YEM) minimal medium under continuous shaking at 150 rpm and a temperature of 27 ± 2 °C. When the OD_{620} reached 0.4–0.5 (4–5 days), genistein (Sigma Chemical Co. MO, USA) was added to a final concentration of 5 μM and the culture was incubated for an additional 48 h. The culture was then be extracted with 0.4 volumes of HPLC-grade 1-butanol (Fisher Scientific Canada) by shaking vigorously for 10–15 min and was then allowed to stand overnight. The organic fraction was then separated and completely dried under vacuum, in a rotary evaporator (Yamato, New York, USA). The resulting material was dissolved in 18% aqueous acetonitrile and this constituted the LCO extract that was fractionated by HPLC (Fisher Scientific, Montreal, Canada). From time-to-time the chemical identity of the LCO was confirmed by FAB–mass spectroscopy and MALDI–TOF mass spectroscopy (as described by Soulemanov *et al.*, 2002a).

3.3.2 Extraction of Thuricin17

The bacterial strain *B. thuringiensis* NEB17 was cultured in King's Medium B (Atlas, 1995). The initial culture was grown in 250 mL flask containing 50 mL of King's B medium at 25 ± 2 °C while being shaken at 150 rpm on an orbital shaker (Model 5430 Table Top Orbital Shaker) for 48 h. A subculture was prepared from the initial culture by adding 5 mL of this stock solution to 2 L of medium and was incubated for further 48 h as described above. The bacterial population of *B. thuringiensis* NEB17 was measured spectrophotometrically using as Ultrospec 4300 Pro UV/Visible Spectrophotometer (Biochrom Ltd, Cambridge, UK) at 600 nm (Dashti *et al.*, 1997) and cell-free supernatant (CFS) containing Th17 was prepared by centrifuging the culture at 13000 g for 10 min. Th17 was isolated from these supernatant using HPLC analytical methods (as described by Gray *et al.* 2006b). The obtained Th17 was purified several times, until single HPLC peak was isolated.

3.3.3 Germination assays

Germination experiments were conducted under controlled environment conditions in germination chambers (Model TC30, Controlled Environments Ltd., Winnipeg, Canada) provided by the Department of Plant Science, Macdonald Campus McGill University. Research already conducted in our laboratory has established that the growth stimulatory effects of Th17 and LCO are greater under stress and that, for soybean, the effects are slight if there is no stress. For soybean the optimum temperature for growth and nitrogen fixation is around 25 – 30 °C (Jones and Tisdale, 1921). Studies have shown that low RZT decrease the growth and nitrogenase activity (Jones and Tisdale, 1921; Hardy *et al.*, 1968; Roughley and Date, 1986; Legros and Smith, 1994; Lynch and Smith, 1994). Thus, the experiments were carried out in this work involved both moderately stressful low temperature (22 ± 2 °C) and severely stressful low temperature (15 ± 2 °C) conditions. The hypothesis was that the beneficial effects of the two signal compounds would be greater under severe stress than moderate stress. Germination was carried out under dark conditions for both temperatures. Soybean (*Glycine max* variety Absolute RR, provided by Belcan, Canada) was used to determine the effects of Th17 and LCO on the progression of seed germination. The treatments were comprised of two concentrations of Th17 (10^{-9}

and 10^{-11} M) and two concentrations of LCO (10^{-6} and 10^{-8} M) plus a distilled water control treatment. Seeds were screened for uniformity of size and overall condition (no obvious damage to the seeds). Ten seeds were placed in each 9 cm sterile petri-plate. The bottom of each plate was lined with filter paper (Qualitative P8, 9 cm diameter, Fisher Scientific). Four milliliters of treatment solution were introduced into each Petri plate and each plate was then sealed using parafilm. The parafilm was cut in 1 cm wide strips and stretched to cover the circumference of the petri plate, at the point where the top and bottom parts are joined. The resulting sets of perti plates, with seeds and added treatment solutions, were incubated under dark conditions in a germination chamber at the appropriate temperature.

The experiment was organized following a completely randomized design (CRD) with 5 replications of each treatment. The number of germinated seeds was counted every 6 h beginning 24 h after establishment of the experiment. Each set of experiments was repeated four times.

3.3.4 Greenhouse experiments

The greenhouse experiments were conducted in greenhouse provided by the Macdonald Campus of McGill University. Soybean (*G. max* variety 91M01, Pioneer) was used to determine the effects of treatments on emergence and early growth under greenhouse conditions. Three treatments were used, one concentration of Th17 (10^{-10} M), one concentration of LCO (10^{-7} M) and water, as a negative control.

The experiment was conducted in pots with a 20 cm top diameter and 14 cm bottom diameter, and 16.5 cm deep. Each pot was filled with an equal amount of agromix, and to a uniform level. Ten seeds were planted in each pot at the depth of 2 cm. They were inoculated with bacterial suspensions of *B. japonicum* (10^8 colony forming cells per mL of inoculum (cfu mL^{-1}). The growth conditions were set at 22 ± 2 °C, 75% relative humidity with a 16 h photoperiod. After establishing the experiment, the pots were watered as required with equal amounts of nitrogen-free Hoagland's solution (Hoagland and Arnon, 1950).

Data were collected on time to emergence of the seeds. When all the seeds were emerged, the plants were thinned, keeping only one representative plant in each pot. The plants were harvested when they reached the pod stage (R5 stage) (Fehr *et al.*, 1971) and data were collected on total height, number of trifoliate leaves, leaf area, fresh weight, total number of pods, shoot dry weight, pod dry weight, root dry weight, number of nodules and nodule dry weight. Leaf area was measured using an image analysis leaf area meter (Delta-T Devices, Cambridge, UK). For dry weight of plants, nodules and roots, the samples were kept separately in paper bags in a drying oven for 3 days at 55-60 °C, by which time they had reached a constant weight.

The experiment was organized following completely randomized design (CRD) with 5 replications of each treatment.

3.3.5 Field trials

Field experiments were conducted for 2 years, in 2010 and 2011, at the E.A. Lods Agronomy Research Center (45° 25′ 45″N latitude and 73° 56′ 00″ longitude) of the Macdonald Campus of McGill University, Ste- Anne-de-Bellevue, Quebec, Canada, from May to September. In 2010, one field experiment was conducted while in 2011 two field trials were conducted, again, to determine the effects of Th17 and LCO on soybean growth and development under field conditions. In 2010, the soybean was planted on May 12 and we expected at least relatively low temperatures during seedling emergence. In 2011 two soybean trials were planted at separate dates: the first trial was started on June 3, 2011 and the second on June 22, 2011. During first 2011 trial, we were trying to capture temperature lower than optimum growing temperatures for soybean seeds (low temperature stress for soybean).

The experiments were organized following a Randomized Complete Block Design (RCBD), in both years, with four blocks (replicates); each block contained seven plots (treatments) in 2010 and eight plots in 2011. Each block measured 4 x 18 m, with a 1.3 m wide pathway between adjacent blocks. Each plot was comprised of nine rows of soybean plants; rows within plots were 18 cm apart.

Soybean (*G. max* variety 91M01, Pioneer) was used in all field trials. Three concentrations of Th17 (10^{-9}, 10^{-10} and 10^{-11} M) and LCO (10^{-6}, 10^{-7} and 10^{-8} M) were used. A water treatment was included as a negative control. Optimize, a commercial product (sold by EMD Crop BioScience, Milwaukee; www.emdcropbioscience.com/homepage.cfm) containing an LCO technology developed in our laboratory, was used as a positive control in the 2011 trials. The field site was plowed to a depth of 20 cm in the autumns of 2009 and 2010 and harrowed during springs of 2010 and 2011, to provide a good seedbed. The fertiliser was applied and then the site was harrowed before sowing the seeds. Fertilizer was applied as 4:20:20 NPK at 250 kg ha^{-1}. Following fertilizer application the rows were prepared using a Plot Man seeder (Swift Current Saskatchewan, Canada). Once the rows were established sowing was done manually. The seeds were sown (approximately 660,000 seeds ha^{-1}) into the open rows and then covered, immediately, using a garden hoe.

The soybean seeds were soaked in the treatment solution for 20 min. prior to planting and then air-dried. After applying the treatment solutions, the seeds were treated with the commercially available Histick N/T soybean inoculant (Becker Underwood, Saskatoon) containing 2×10^9 viable *B. japonicum* cells and 1×10^8 viable cells of *Bacillus subtilis* (MBI600 strain) per gram of inoculum. The inoculant was applied at 400 g (125 kg of soybean seed)$^{-1}$.

Data were collected on emergence until no new seedlings were seen to be appearing, to determine the percentage emergence. During the course of the growing season, data were collected at three crop growth stages. The first sampling was done at the mid-vegetative stage (V(N) stage, Fehr *et al.*, 1971), more than 3 nodes on main stem beginning with the unifoliate node, second at the flowering stage (R2 or R3 stage), and last sampling at the mature or harvest stage (R8 stage, when 95% of the pods are brown, harvest maturity).

For each sampling, 10 plants were selected randomly from each plot. At first sampling, data were collected on fresh weight of the plants, number of trifoliate leaves, leaf area and the dry weight of the plants. At the flowering stage, data on fresh weight

per plant, number of trifoliate leaves, leaf area, dry weight, number of nodules, dry weight of nodules and dry weight of roots were collected.

When the soybean plants reached harvest maturity (R8 stage), 10 plants were selected randomly from each plot to collect data on yield components. The rest of the plot was harvested with a small-plot combine (Wintersteiger Classic, Utah, U.S.A.) to determine the total yield from each plot. From the last sampling, the following data on yield components were collected: pods per plant, seeds per plant, weight of seeds per plant and 100-seed weight.

3.3.6 Data analysis

Data were analyzed using PROC MIXED (SAS Institute, 2009) for the field trials while PROC GLM was used to analyse data from the germination and greenhouse experiments. Treatment means from the germination chamber and greenhouse experiments were compared with a protected least significant difference (LSD) test at $P < 0.05$. Treatment means from the field study were compared using a Scheffe's multiple comparisons test at $P < 0.05$.

3.4 Results

3.4.1 Effect of Th17 and LCO on soybean germination

The results indicated that with the use of Th17 and LCO, the germination process can be accelerated for soybean under both evaluated temperature conditions, although the effects were greater when the conditions were severely stressful.

3.4.1.1 Effect of Th17 and LCO on soybean germination at a moderately stressful temperature (22 ± 2 °C)

At a moderately stressful temperature (22 °C), both Th17 and LCO treatments resulted in higher % germination than the control treatment. ANOVA showed that the effect of these treatments on germination of soybean was highly significant at 24 h after treatment ($P < 0.01$) and that the effect declined over time; the statistical significance level at 30 h was $P < 0.06$ and at 36 h it was $P > 0.1$ (Table 1).

An LSD test ($p < 0.5$) showed that both concentrations of Th17 and one concentration of LCO (10^{-6}) resulted in germination rates that were greater than the

control at 24 and 30 h. When treatment means were compared at 24 h both concentrations of Th17 (10^{-9} and 10^{-11} M) and LCO (10^{-6} M) caused almost 10% increases in the germination, as compared to the control, while at 30 h Th17 (10^{-9} and 10^{-11} M) and LCO (10^{-6} and10^{-7} M) resulted in germination % that was 15, 17, 13 and 6 %, respectively, greater than the control (Table 3). At 30 h after treatment, Th17 (10^{-9} and 10^{-11} M) and LCO (10^{-6} M) caused 15, 17 and 13% numerical increases in germination, as compared to the water control.

3.4.1.2 Effect of Th17 and LCO on soybean germination at severely stressful low temperature (15 ± 2 °C)

When the temperature was very stressfully low (15 °C), treatment with Th17 and LCO enhanced the germination of soybean. Germination at 15 °C began much later than at 22 °C. Both Th17 and LCO treatments accelerated the germination relative to the water control.

ANOVA indicated that at 36 h the effect of treatment was not significant, but from 48 to 72 h the effect of treatment was significant ($P < 0.01$) (Table 2). An LSD comparison of the means at 54, 60 and 72 h, detected increased % germination due to all Th17 and LCO treatments. Treatment with Th17 (10^{-9}) and LCO (10^{-8}) resulted in more pronounced effects, with higher % germinations than other treatments.

Figure (2) shows clearly that that both concentrations of Th17 and LCO enhanced the germination of soybean. The greatest differences occurred at 54 and 60 h after treatment. At 54 h ($P < 0.038$) LCO (10^{-6} M) and Th17 (10^{-8} M) increased seed germination by 19 and 11 %, respectively, as compared to the control, while Th17 at 10^{-9} and 10^{-11} M increased germination by 15 and 14 %, respectively. At 60 h ($P < 0.006$), Th17 at 10^{-9} and 10^{-11} M increased germination by 25 and 16 %, respectively, over the control (Table 3).

When the means were compared, at 54, 60 and 72 h, using an LSD test, all the Th17 and LCO treatments resulted in higher % germination levels than the control. Thuricin 17 (10^{-9} M) and LCO (10^{-8} M) resulted in more pronounced effects than the other signal compound treatments.

3.4.2 Effect of Th17 and LCO on soybean growth and development under greenhouse conditions

The results showed that both Th17 (10^{-10}) and LCO (10^{-7}) treatments had positive effects on the dry weight of pods and roots. However they did not have any effect on leaf area, number of trifoliate leaves, number of pods and total dry weight of the shoots.

Use of Th17 and LCO increased the total biomass of the pods ($P < 0.01$) and dry weight of roots ($P < 0.01$). Th17 and LCO increased the pod weight to 2.13 and 1.54 g plant^{-1}, resulting in an increase of 79 and 30%, respectively, over the controls (Table 4).

Root dry weight data indicated that both Th17 and LCO treatment resulted in bigger roots ($P < 0.015$) than the control plants. LCO treated plants had heavier roots (561.1 mg plant^{-1}) than Th17 treated (350.4 mg plant^{-1}) and control (288.5 mg plant^{-1}) plants (Table 4). Similarly, Th17 treatment had a negative effect on the number of nodules ($P < 0.01$). Th17 treated plants had the lowest number of nodules (32 per plant); while LCO treatment resulted in 61 per plant and control treatment resulted in 55 per plant. However, the dry weight of nodules was highest for Th17 treated plants, at 74 mg per plant, while LCO and control treatments resulted in 48 and 56 mg per plant, respectively. There were no differences among treatments for other growth variables, such as leaf area, number of pods and dry biomass, although these were generally numerically greater for both Th17 and LCO treated plants (Table 4).

3.4.3 Effect of Th17 and LCO on soybean growth and yield under field condition

Trials were conducted for two years to examine the effects of the Th17 and LCO under field conditions, as the experiments conducted under growth chamber and greenhouse conditions showed positive effects of signal compounds on growth and development of soybean plants. One trial was conducted in 2010 and two trails were conducted in 2011. The 2011 trials were planted at different dates as we were trying to determine the effects of low temperature stress during the germination of soybean and it was hoped that comparison between the earlier seeded first trial and the later seeded second trial in 2011 would provide that insight. Daily temperature (minimum and

maximum reading) and precipitation values for the months of May-June 2010 and June-July 2011 are presented in Figures 7 (a), 7 (b), 8 (a) and 8 (b), respectively.

Data were collected on various growth and development variables, as indicated in the materials and methods, from all the trials of 2010 and 2011. The results from 2010 showed that the LCO and Th17 treatments had some effects on soybean plants during flowering stage. LCO (10^{-6} and 10^{-7} M) and Th17 (10^{-11} M) treatments increased ($P < 0.05$) leaf area while all LCO treatments and Th17 (10^{-11} M) increased ($P < 0.05$) total plant biomass. LCO (10^{-6} M) and Th17 (10^{-10} M) resulted in a higher number ($P < 0.1$) of nodules per plant and only LCO (10^{-8} M) treatment resulted in increased ($P < 0.001$) nodule dry biomass, as compared to the water control (Table 5).

However, there were no significant differences between the treatments and the control for measured variables in either of the 2011 trials. All treatments resulted in similar mean values for all sampled variables. In addition to water, as a negative control, we used a commercially available LCO containing product (Optimize) as a positive control. Under the conditions of these experiments there were also no effects of Optimize when compared to the water-only control.

3.5 Discussion

I have shown that both Th17 (10^{-9} and 10^{-11} M) and LCO (10^{-6} and 10^{-8} M) treatments accelerated the germination of soybean relative to the water control under controlled environmental condition of growth chamber. When the temperature was severely stressful (15 ± 2 °C), both Th17 and LCO treatments had greater effects on germination, as compared to the moderately stressful temperature of 22 ± 2 °C. This indicated that under more stressful conditions, the effect of the signal treatments were greater. These results showed clearly that both Th17 and LCO can directly enhance plant growth, which supported the previous findings of Lee *et al.* (2009) for Th17 and Prithiviraj *et al.* (2003) for LCO.

For soybean, the germination process is much slower at 15 °C than at 22 °C. It has been shown that a low root zone temperature (RZT) not only delays germination but also decreases nodulation and nodule function (Lynch and Smith, 1994). The time

between soybean inoculation and the start of nitrogen fixation was delayed by 2 days for each degree decrease in temperature between 25 and 17 °C, and this delay in time increased to one week for each degree decrease in temperature between 17 and 15 °C (Zhang *et al.*, 1995). As indicated above, with the use of Th17 and LCO treatments, the soybean germination process can be enhanced at low RZT (15 °C). Based on various experiments now being carried out in our laboratory, we can now say that Th17 and LCO treatments show greater effects under stressful conditions, such as salinity, low temperature or drought (unpublished data).

In all our germination assays, except for the imposed moderate and severe low temperature stress, the experiments were carried out under otherwise ideal growing conditions, so all the seeds that could germinate eventually did germinate, however, under field conditions this is unlikely to be the case; slower germination often means fewer plants emerge. Hence, under such stressful conditions, Th17 and LCO can help crops to overcome stress and maintain the good seed germination and, finally, good crop stand.

We further tested the effects of Th17 and LCO on soybean growth under greenhouse conditions. In this experiment, the plants were grown in pots until they reached the pod stage (R5 stage, Fehr *et al*. 1971) and data were collected on various growth variables. The results showed that both Th17 (10^{-10} M) and LCO (10^{-7} M) treatments increased the dry weight of pods and roots. This result is consistent with those of Khan *et al.* (2011) who found that LCO increases root growth of Arabidopsis. Although all treatments, including the water control, had similar numbers of pods per plant, Th17 (10^{-10} M) and LCO (10^{-7} M) treatments resulted in increased pod dry weight per plant (Table 4). Th17 and LCO treatment increased the dry weight of pods by 79 and 30 %, respectively, as compared to the water control. This indicated that all the three treatments resulted in similar numbers of pods, but the pods produced were larger for Th17 and LCO treatments. In addition, Th17 decreased the number of nodules (32 per plant) as compared to LCO (61 per plant) and the water control (55 per plant), but it resulted in bigger nodules and more final nodule biomass than the water control and LCO treated plants (Table 4). These results generally support previous

findings from our laboratory. When Th17 was applied to either leaves or roots, they increase the leaf area, plant dry matter accumulation and photosynthetic rate (Lee *et al.*, 2009). In those experiments Th17 was applied 3 times at different growing stages at the rate of 1 mL per plant. Similarly, LCO treatment increased leaf area, root and shoot length, and root and shoot dry matter (Prithiviraj *et al.*, 2003). In addition, in this experiment LCO treatments were applied at the rate of 20 mL per plant every 2 days for 15 days, at which time the plants were harvested. But in our experiment the Th17 treatment was applied to seeds only once. Here, with only one application of Th17 and LCO, we were able to see the effect of treatment on soybean plants. Thus, these findings indicate that only one application, acute exposure, of these signal compounds is also effective and there is potential for commercial application of Th17 as a crop growth enhancer. With an acute exposure, the efficacy of the treatments can be enhanced, with the use of various technologies developed for seed treatment.

Growing conditions in the field are unlikely to be as ideal as in the greenhouse and growth chambers. There are various factors in the field, which cannot be controlled and which potentially have direct effects on how the treatments work. Thus, the field trials were conducted to examine the effect of Th17 and LCO. As indicated above, two years of field trials were conducted, one trial in 2010 and two trials in 2011. In 2010, the trial was started in early May so that the low temperature stress (around 15 °C) could be captured during emergence of soybean. Similarly, in 2011 two trials were conducted at two different dates as I was trying to determine the effects of low temperature stress during the germination of soybean seeds in the first trial, following from our germination assay work. The 2010 field trial was started on May 12 and the average maximum and minimum daily temperature for two week after sowing seeds was 23 and 9.5 °C respectively. Due to higher temperatures, around 23 °C, we were not able to capture the low temperature stress effects. In addition, there was very little precipitation during May 2010, after seeding the seeds (Figure 7 (b)). The emergence of the soybean plants was very poor (~45-65 %) at one month after seeding. We were able to observe some differences among the treatments at the flowering stage but these differences did not persist until the final harvest stage. LCO and Th17 treatments

increased the leaf area, total plant biomass, plus number and biomass of nodules, which supported the previous findings of Lee *et al.* (2009) and Prithiviraj *et al.* (2003).

Similarly in 2011, I started the first trial on June 3rd and was expecting the soil temperatures to be in the range of 15 to 17 °C during the following week, based on long-term norms. However, the temperature conditions were not as hoped and it was generally above 22 °C for the week following seeding. Because of unanticipated weather conditions we were not able to capture the low temperature stress effects during germination.

The results of both field trails in 2011 showed no differences among the treatments. However, there were also no negative effects on the crop growth and development with the use of Th17 and LCO. In addition, there were not any stresses present, such as low temperature stress, following seeding. Previous experiments with Th17 under field conditions showed that Th17 enhances germination of corn seeds but, at that time the temperature during the germination was around 13 to 15 °C (He, 2009). I note that the commercial product, Optimize also did not have positive effects in these experiments. It is known that Optimize treatment does not always result in beneficial effects under field conditions, and my data, plus that of others in our laboratory, suggest that is probably due to the presence or absence of stress during key times during crop development. Finally, with the use of LCO, the yield of soybean was increased up to 25 % (Jose Almaraz *et al.*, 2007) under field conditions, but in this experiment the LCO was repeatedly sprayed on soybean leaves, constituting a chronic application.

The increase in plant growth variables, either germination, dry matter accumulation in roots or pods, could be because of hormone like effects of LCOs (Prithiviraj *et al.*, 2003). Nod factors are known to induce the cell division (Schlaman *et al.*, 1997) not only in legumes but also in non-legumes (De Jong *et al.*, 1993; Dyachok *et al.*, 2000). LCO like molecules also stimulate early somatic embryo development in Norway spruce (Dyachok *et al.*, 2002). Enhanced germination and seedling growth, along with the mitogenic nature of LCOs (Spaink *et al.*, 1991; Truchet *et al.*, 1991), suggest accelerated meristem activity. This may lead to increased sink demand and the

observed increases in mobilization of seed reserves (Prithiviraj *et al.*, 2003) and increased photosynthetic rates (Jose Almaraz *et al.*, 2007) for more developed plants; both of which lead to increased growth (Khan *et al.*, 2008). LCOs are known to activate specific plant genes and act as mitogens (Long, 1989; Dénarié *et al.*, 1996; Perret *et al.*, 2000; Patriarca *et al.*, 2004). My results are consistent with this view however, the interaction with stress adds an additional component to the earlier findings, one that is now being clearly demonstrated, but that is not well understood at this time.

3.6 Conclusions

In this study we investigated the effects of Th17 and LCO treatments on soybean growth under a range of growing conditions extending from growth chambers to the field. This is the first study conducted to determine the effect of acute exposure of Th17 on soybean growth under greenhouse and field conditions. Soybean is sub-tropical in origin and requires temperatures of 25 – 30 °C for the optimum growth. Here I have demonstrated that the use of Th17 and LCO helps to overcome both moderate (22 °C) and severe (15 °C) low temperature stress effects during the emergence of soybean seedlings, and to maintain good early growth thereafter. As Th17 is a newer compound, in comparison to LCO, which is already in the market place, Th17 had been compared with LCO throughout these experiments. Collectively, these studies showed that Th17 has potential to be used as a commercial technology, as a crop growth enhancer. However, there is still a lot of characterization and research required, in order to better understand the mode of action and range of crops being influenced.

Table 1. ANOVA and LSD test for the effect of Th17 and LCO on germination of soybean at 22 °C. The t groupings obtained from LSD analysis are given only for those variables which were significantly different (at $P < 0.05$). Means associated with the same letter are not different by the ANOVA protected LSD test. (10^{-6}, 10^{-8}, 10^{-9} and 10^{-11} indicate molar concentrations of either LCO or Th17)

Hours after treatment	**ANOVA**		**LSD for comparisons among treatments**		
	Source	P value	Treatments	Means ± SE	t grouping
24 h	Treatment	0.017**	LCO (10^{-6})	33 ± 3.6	A
			LCO (10^{-8})	19 ± 3.6	C
			Th17 (10^{-9})	30 ± 3.6	AB
			Th17 (10^{-11})	31 ± 3.6	A
			Control	20 ± 3.6	C
30 h	Treatment	0.0683***	LCO (10^{-6})	63 ± 4.6	AB
			LCO (10^{-8})	56 ± 4.6	AB
			ThH17 (10^{-9})	65 ± 4.6	A
			Th17 (10^{-11})	67 ± 4.6	A
			Control	50 ± 4.6	B
36 h	Treatment	0.1559 NS	LCO (10^{-6})	86 ± 3.6	
			LCO (10^{-8})	89 ± 3.6	
			Th17 (10^{-9})	93 ± 3.6	
			Th17 (10^{-11})	91 ± 3.6	
			Control	81 ± 3.6	

** indicates significant differences at 0.05 probability level, *** indicates significant differences at 0.1 probability level and NS indicates no significant difference at the 0.1 probability level. SE = standard error of the mean. Means presented in the above table are the average of 20 replicates.

Table 2. ANOVA and LSD test for the effect of Th17 and LCO on germination of soybean at 15 °C. The t groupings obtained from LSD analysis are given only for those variables which were significantly different (at $P < 0.05$). Means associated with the same letter are not different by the ANOVA protected LSD test. (10^{-6}, 10^{-8}, 10^{-9} and 10^{-11} indicate molar concentrations of either LCO or Th17)

Hours after treatment	**ANOVA**		**LSD for comparison among treatments**		
	Source	P value	Treatment	Means ± SE	t grouping
48 h	Treatment	0.008*	LCO (10^{-6})	55 ± 3.9	A
			LCO (10^{-8})	42 ± 3.9	B
			Th17 (10^{-9})	43 ± 3.9	B
			Th17 (10^{-11})	42 ± 3.9	B
			Control	34 ± 3.9	B
54 h	Treatment	0.038**	LCO (10^{-6})	65 ± 4.3	A
			LCO (10^{-8})	57 ± 4.3	AB
			Th17 (10^{-9})	61 ± 4.3	A
			Th17 (10^{-11})	60 ± 4.3	A
			Control	46 ± 4.3	B
60 h	Treatment	0.0006*	LCO (10^{-6})	89 ± 4.2	A
			LCO (10^{-8})	75 ± 4.2	BC
			Th17 (10^{-9})	90 ± 4.2	A
			Th17 (10^{-11})	81 ± 4.2	AB
			Control	65 ± 4.2	C
72 h	Treatment	0.03**	LCO (10^{-6})	100 ± 2.2	A
			LCO (10^{-8})	97 ± 2.2	A
			Th17 (10^{-9})	97 ± 2.2	A
			Th17 (10^{-11})	98 ± 2.2	A
			Control	90 ± 2.2	B

*indicates high level of significant differences at the 0.001 probability level and ** indicates significant differences at the 0.05 probability level. SE = standard error of the mean. Means presented in the above table are the average of 20 replicates.

Table 3. Percentage increase in germination due to treatment with Th17 and LCO, over the water control at 22 and 15 °C

Growing temperature	% increase in germination over control at different Time points				
22 ± 2°C	**Treatment**	**24 h**	**30 h**	**36 h**	
	Control	-	-	-	
	LCO (10^{-6})	13	13	5	
	LCO (10^{-8})	-1	6	8	
	Th17 (10^{-9})	10	15	12	
	Th17 (10^{-11})	11	17	10	
15 ± 2°C	**Treatment**	**36 h**	**48 h**	**54 h**	**60 h**	**72 h**
	Control	-	-	-	-	-
	LCO (10^{-6})	7	21	19	24	10
	LCO (10^{-8})	5	8	11	10	7
	Th17 (10^{-9})	4	9	15	25	7
	Th17 (10^{-11})	6	8	14	16	8

Means of % germination presented in the above table are the average of 20 replicates

Table 4. ANOVA and LSD test for the effect of Th17 and LCO on growth and development of soybean under greenhouse conditions. The t groupings obtained from LSD analysis are given only for those variables which were significantly different (at $P < 0.05$). Means associated with the same letter are not different by the ANOVA protected LSD test. (10^{-7} and 10^{-10} indicate molar concentrations of LCO and Th17, respectively)

Parameter	**ANOVA**		**LSD for comparison among treatments**		
	Source	**P value**	**Treatment**	**Means ± SE**	**t grouping**
Leaf Area (cm^2)	Treatment	0.464 ^{NS}	Control	166.2 ± 42.4	
			LCO (10^{-7})	236.0 ± 42.4	
			Th17 (10^{-10})	228.4 ± 42.4	
Number of Pods	Treatment	0.839 ^{NS}	Control	8.8 ± 1.6	
			LCO (10^{-7})	9.4 ± 1.6	
			Th17 (10^{-10})	10.2 ± 1.6	
Total Dry weight (g)	Treatment	0.194 ^{NS}	Control	2.24 ± 0.5	
			LCO (10^{-7})	3.20 ± 0.5	
			Th17 (10^{-10})	3.63 ± 0.5	
Dry weight of pods (mg)	Treatment	0.072***	Control	1187.4 ± 262.4	B
			LCO (10^{-7})	1546.9 ± 262.4	AB
			Th17 (10^{-10})	2131.8 ± 262.4	A
Number of nodules	Treatment	0.097***	Control	55.6 ± 9.3	A
			LCO (10^{-7})	61.6 ± 9.3	AB
			Th17 (10^{-10})	32.0 ± 9.3	B
Dry weight of nodules (mg)	Treatment	0.481^{NS}	Control	56.08 ± 14.6	
			LCO (10^{-7})	48.92 ± 14.6	
			Th17 (10^{-10})	74.04 ± 14.6	

Parameter	ANOVA		LSD for comparison among treatments		
	Source	**P value**	**Treatment**	**Means ± SE**	**t grouping**
Dry weight of root (mg)	Treatment	0.015**	Control	288.5 ± 57.8	B
			LCO (10^{-7})	561.1 ± 57.8	A
			Th17 (10^{-10})	350.4 ± 57.8	A

** indicates significant differences at 0.05 probability level, *** indicates significant differences at 0.1 probability level, and NS indicates no significant difference at the 0.1 probability level. SE = standard error of the mean. Means presented in the above table are the average of 5 replicates.

Table 5. ANOVA and LSD test for the effect of Th17 and LCO on growth and development of soybean under field conditions in 2010. The table consists of the average readings for variables measured at the flowering stage of the soybean plants. The t groupings obtained from LSD analysis are given only for those variables which were significantly different (at $P < 0.05$). Treatments followed by the same letters are not significantly different at 5 % probability level. (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} indicate molar concentrations of either LCO or Th17)

Parameter	**ANOVA**		**LSD for comparison among treatments**		
	Source	P value	Treatment	Means ± SE	t grouping
Leaf Area (cm^2)	Treatment	0.0347**	Control	1093.1 ± 127.9	CD
			LCO (10^{-6})	1466.0 ± 127.9	A
			LCO (10^{-7})	1369.2 ± 127.9	AB
			LCO (10^{-8})	1283.5 ± 127.9	ABC
			Th17 (10^{-9})	1010.3 ± 127.9	D
			Th17 (10^{-10})	1231.2 ± 127.9	BC
			Th17 (10^{-11})	1356.7 ± 127.9	AB
Total Dry weight (g)	Treatment	0.0261**	Control	9.95 ± 0.9	C
			LCO (10^{-6})	12.92 ± 0.9	A
			LCO (10^{-7})	12.46 ± 0.9	AB
			LCO (10^{-8})	11.87 ± 0.9	B
			Th17 (10^{-9})	9.03 ± 0.9	CD
			Th17 (10^{-10})	10.97 ± 0.9	BC
			Th17 (10^{-11})	12.05 ± 0.9	AB
Number of Nodules	Treatment	0.0835***	Control	34.3 ± 3.8	CD
			LCO (10^{-6})	51.1 ± 3.8	A
			LCO (10^{-7})	37.5 ± 3.8	BC
			LCO (10^{-8})	36.3 ± 3.8	BC
			Th17 (10^{-9})	37.0 ± 3.8	BC
			Th17 (10^{-10})	39.6 ± 3.8	B
			Th17 (10^{-11})	37.3 ± 3.8	BC

Parameter	ANOVA		LSD for comparison among treatments		
	Source	P value	Treatment	Means ± SE	t grouping
Dry weight of Nodules (g)	Treatment	0.0004*	Control	0.21 ± 0.02	BC
			LCO (10^{-6})	0.24 ± 0.02	B
			LCO (10^{-7})	0.17 ± 0.02	CD
			LCO (10^{-8})	0.30 ± 0.02	A
			Th17 (10^{-9})	0.13 ± 0.02	D
			Th17 (10^{-10})	0.15 ± 0.02	D
			Th17 (10^{-11})	0.21 ± 0.02	BC

*indicates high level of significant differences, at the 0.001 probability level, ** indicates significant differences at the 0.05 probability level and *** indicates significant differences at the 0.1 probability level. Means presented are the average of 40 replicates.

Table 6. Effect of Th17 and LCO on growth and development of soybean under field conditions (Trial 1, 2011). The table consists of the average reading for different parameter in different growing stages of the soybean plant (at vegetative stage, flowering stage, harvest stage and combine harvest). Means presented are the average of 40 replicates. (Control: water control; Optimize: commercial LCO technology; LCO (10^{-6}): 10^{-6} M LCO; LCO (10^{-7}): 10^{-7} M LCO; LCO (10^{-8}): 10^{-8} M LCO; Th17 (10^{-9}): 10^{-9} M Th17; Th17 (10^{-10}): 10^{-10} M Th17 and Th17 (10^{-11}): 10^{-11} M Th17)

Treatment	**Vegetative Stage**		**Flowering Stage**				
	Leaf Area (Sq. cm)	Dry Weight of Shoot(gm)	Leaf Area (Sq. cm)	Dry Weight of Shoot (gm)	Number of Nodules	Dry Weight of Nodules (mg)	Dry Weight of Root (mg)
Control	317.4 ± 23.8	2.13 ± 0.1	853.6 ± 37.8	7.62 ± 0.4	27.1 ± 2.6	89.28 ± 11.5	938.9 ± 54.9
Optimize	321.4 ± 23.6	2.11 ± 0.1	730.2 ± 38.2	6.35 ± 0.4	32.5 ± 2.6	115.6 ± 11.4	861.6 ± 54.9
LCO(10^{-6})	338.4 ± 23.7	2.24 ± 0.1	784.5 ± 37.8	6.81 ± 0.4	30.7 ± 2.6	99.85 ± 11.4	963.4 ± 54.4
LCO(10^{-7})	322.3 ± 23.7	2.09 ± 0.1	811.2 ± 37.8	7.17 ± 0.4	28.5 ± 2.6	106.6 ± 11.4	948.8 ± 55.5
LCO(10^{-8})	323.7 ± 23.7	2.08 ± 0.1	679.3 ± 39.2	6.06 ± 0.4	32.3 ± 2.6	109.3 ± 11.4	854.8 ± 55.6
Th17 (10^{-9})	317.8 ± 24.0	2.12 ± 0.1	709.2 ± 38.6	6.04 ± 0.4	26.7 ± 2.6	96.68 ± 11.6	852.0 ± 54.9
TH17 (10^{-10})	337.0 ± 23.6	2.25 ± 0.1	681.5 ± 37.8	6.15 ± 0.4	30.8 ± 2.6	104.7 ± 11.4	764.8 ± 53.9
Th17 (10^{-11})	317.7 ± 23.7	2.05 ± 0.1	791.0 ± 38.2	6.97 ± 0.4	28.8 ± 2.6	111.8 ± 11.5	915.8 ± 55.5

Treatment	Harvest stage			Final Harvest with combine	
	Dry Weight of seed per plant (gm)	Number of seeds per plant	Number of Pods per plant	Yield per plot (combine) gm	1000 Seed Weight (gm)
Control	9.61 ± 0.6	53.93 ± 2.8	23.7 ± 1.4	2046.6 ± 150.6	175.2 ± 1.6
Optimize	8.80 ± 0.6	50.28 ± 2.8	22.4 ± 1.4	2076.3 ± 150.6	175.8 ± 1.6
LCO(10^{-6})	6.60 ± 0.6	53.10 ± 2.8	24.4 ± 1.4	1918.1 ± 150.6	175.7 ± 1.6
LCO(10^{-7})	9.58 ± 0.6	54.21 ± 2.8	23.2 ± 1.4	1966.4 ± 150.6	172.0 ± 1.6
LCO(10^{-8})	9.80 ± 0.6	55.75 ± 2.8	25.5 ± 1.4	1897.5 ± 150.6	173.9 ± 1.6
Th17 (10^{-9})	9.34 ± 0.6	51.65 ± 2.8	21.8 ± 1.4	2139.8 ± 150.6	174.4 ± 1.6
TH17 (10^{-10})	8.85 ± 0.6	49.68 ± 2.8	21.6 ± 1.4	2191.1 ± 150.6	173.7 ± 1.6
Th17 (10^{-11})	10.06 ± 0.6	57.16 ± 2.8	26.5 ± 1.4	1772.8 ± 150.6	174.7 ± 1.6

Table 7. Effect of Th17 and LCO on growth and development of soybean under field conditions (Trial 2, 2011). The table consists of the average reading for different parameter in different growing stages of the soybean plant (vegetative stage, flowering stage, harvest stage and combine harvest). Means presented are the average of 40 replicates. (Control: water control; Optimize: commercial LCO technology; LCO (10^{-6}): 10^{-6} M LCO; LCO (10^{-7}): 10^{-7} M LCO; LCO (10^{-8}): 10^{-8} M LCO; Th17 (10^{-9}): 10^{-9} M Th17; Th17 (10^{-10}): 10^{-10} M Th17 and Th17 (10^{-11}): 10^{-11} M Th17)

Treatment	**Vegetative Stage**		**Flowering Stage**				
	Leaf Area (Sq. cm)	Dry Weight of Shoot (gm)	Leaf Area (Sq. cm)	Dry Weight of Shoot (gm)	Number of Nodules	Dry Weight of Nodules (mg)	Dry Weight of Root (mg)
Control	280.8 ± 20.1	1.88 ± 0.1	679.4 ± 32.2	6.6 ± 0.3	29.3 ± 3.1	101.9 ± 14.0	1086.0 ± 60.6
Optimize	285.4 ± 20.1	1.95 ± 0.1	666.9 ± 32.2	6.3 ± 0.3	31.5 ± 3.1	98.4 ± 14.1	943.2 ± 60.6
LCO(10^{-6})	291.8 ± 20.1	2.00 ± 0.1	653.9 ± 32.2	6.1 ± 0.3	25.3 ± 3.1	82.5 ± 14.0	991.0 ± 61.0
LCO(10^{-7})	281.4 ± 20.1	1.98 ± 0.1	670.1 ± 32.2	6.3 ± 0.3	30.1 ± 3.1	93.4 ± 14.1	950.7 ± 61.9
LCO(10^{-8})	272.0 ± 20.1	1.89 ± 0.1	649.9 ± 32.2	6.2 ± 0.3	29.8 ± 3.1	94.8 ± 14.0	934.2 ± 60.6
Th17 (10^{-9})	295.0 ± 20.1	2.04 ± 0.1	597.8 ± 32.2	5.9 ± 0.3	29.0 ± 3.1	90.4 ± 14.0	871.3 ± 61.9
TH17 (10^{-10})	301.5 ± 20.1	2.16 ± 0.1	646.4 ± 32.2	6.4 ± 0.3	23.5 ± 3.1	83.1 ± 14.0	937.4 ± 61.5
Th17 (10^{-11})	290.9 ± 20.1	2.09 ± 0.1	682.0 ± 32.2	6.7 ± 0.3	31.4 ± 3.1	110.5 ± 14.0	1018.5 ± 60.6

Treatment	Harvest stage			Final Harvest with combine	
	Dry Weight of seed per plant (gm)	Number of seeds per plant	Number of Pods per plant	Yield per plot (combine) gm	1000 Seed Weight (gm)
Control	8.4 ± 0.6	48.5 ± 3.0	22.1 ± 1.1	1882.1 ± 71.1	164.8 ± 2.0
Optimize	8.7 ± 0.6	50.3 ± 3.0	21.0 ± 1.1	1908.2 ± 71.1	164.3 ± 2.0
LCO(10^{-6})	8.7 ± 0.6	51.3 ± 3.0	21.9 ± 1.1	1961.8 ± 71.1	169.3 ± 2.0
LCO(10^{-7})	8.2 ± 0.6	48.8 ± 3.0	20.3 ± 1.1	1924.9 ± 71.1	169.3 ± 2.0
LCO(10^{-8})	7.1 ± 0.6	42.1 ± 3.0	18.1 ± 1.1	1947.6 ± 71.1	171.2 ± 2.0
Th17 (10^{-9})	8.8 ± 0.6	52.3 ± 3.1	21.8 ± 1.1	1825.6 ± 71.1	171.0 ± 2.0
TH17 (10^{-10})	8.1 ± 0.6	46.4 ± 3.1	20.1 ± 1.1	1932.5 ± 71.1	171.9 ± 2.0
Th17 (10^{-11})	8.4 ± 0.6	48.4 ± 3.0	21.2 ± 1.1	1822.5 ± 71.1	168.4 ± 2.0

Figure 1: Effects of soybean seed treatment with either Th17 or LCO on percent germination over time at 22 °C. The Y-axis indicates the % of germination and the X-axis indicates time points when the observations were made. Each point in the graph is the average reading from 20 replicates and is given +/- standard error. L (-6): 10^{-6} M LCO, L (-8): 10^{-8} M LCO, T (-9): 10^{-9} M Th17 and T (-11): 10^{-11} M Th17.

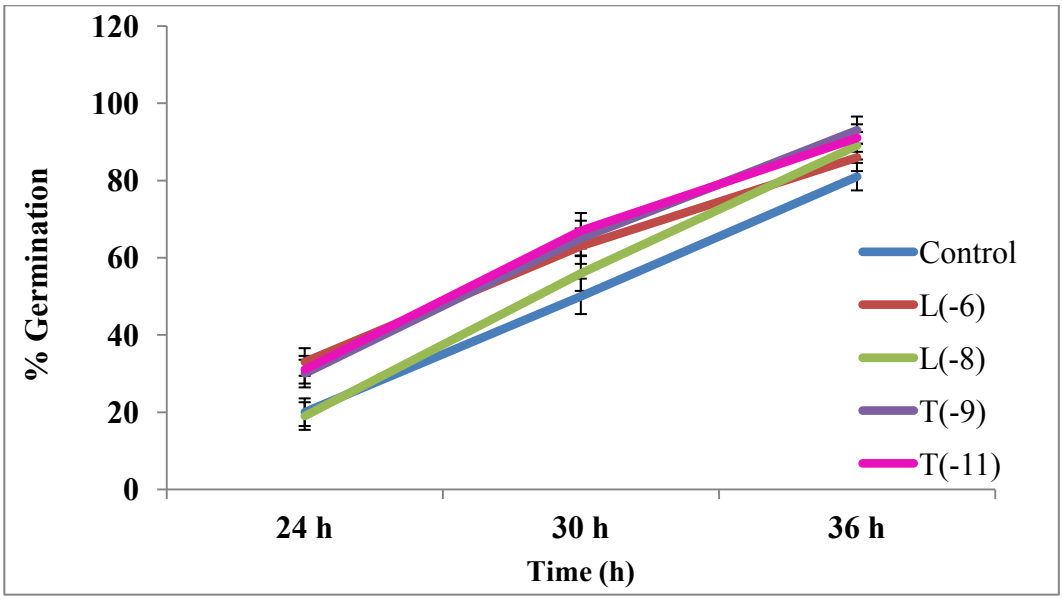

Figure 2: Effects of soybean seed treatment with either Th17 or LCO on percent germination over time at 15 °C. The Y-axis indicates the % of germination and the X-axis indicates time points when the observations were made. Each point on the graph is the average of 20 replicates and is given +/- standard error. L (-6): 10^{-6} M LCO, L (-8): 10^{-8} M LCO, T (-9): 10^{-9} M Th17 and T (-11): 10^{-11} M Th17.

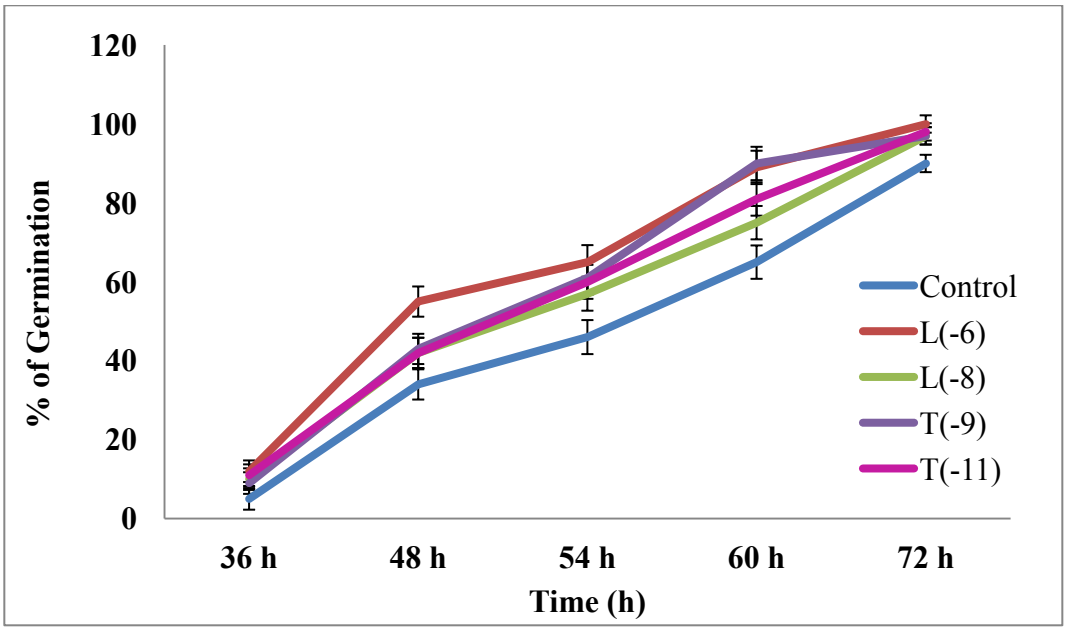

Figure 3: Effects of soybean seed treatment with either Th17 or LCO on growth and development of soybean under greenhouse conditions. Each bar represents the mean, based on 5 replicates and is given +/- standard error. LCO A: 10^{-6} M LCO, Th17A: 10^{-10} M Th17 and Control: water control.

3(a) Effects of LCO (10^{-7}) and Th17 (10^{-10}) on leaf area (cm^2) of soybean plants (R_8 development stage). The Y-axis indicates leaf area (cm^2).

3(b) Effects of LCO (10^{-7}) and Th17 (10^{-10}) on dry weight of pods per plant (mg) of soybean plants (R_8 development stage). The Y-axis indicates dry weight of pods per plant (mg).

3(c) Effects of LCO (10^{-7}) and Th17 (10^{-10}) on numbers of nodule and dry weight of nodules per plant (mg) of soybean plants (R_8 development stage). The Y-axis indicates numbers of nodule and dry weight of nodules per plant (mg).

3(d) Effects of LCO (10^{-7}) and Th17 (10^{-10}) on dry weight of root per plant (mg) of soybean plants (R_8 development stage). The Y-axis indicates dry weight of root (mg).

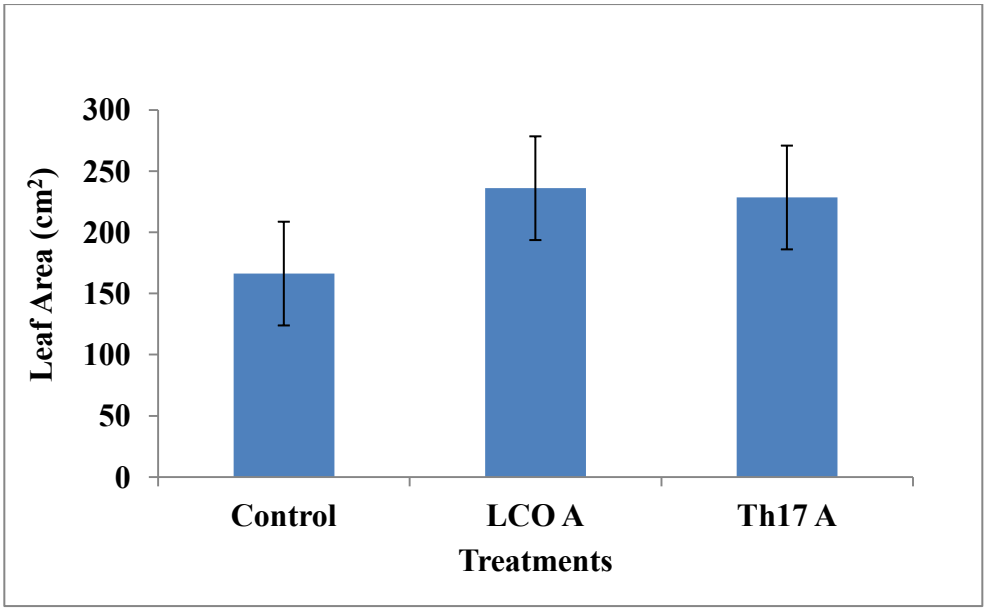

Figure 3 (a)

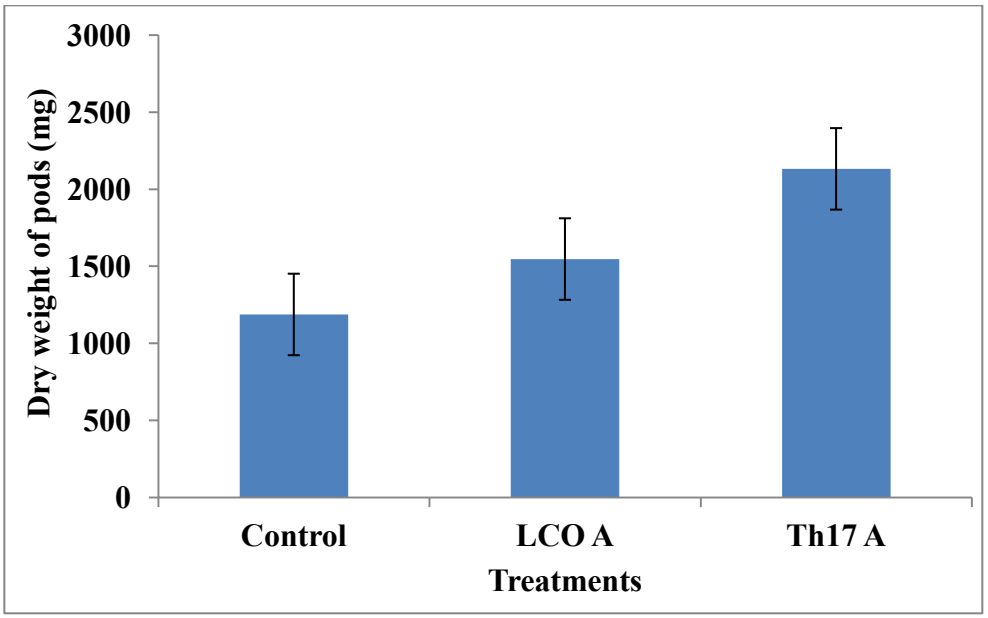

Figure 3 (b)

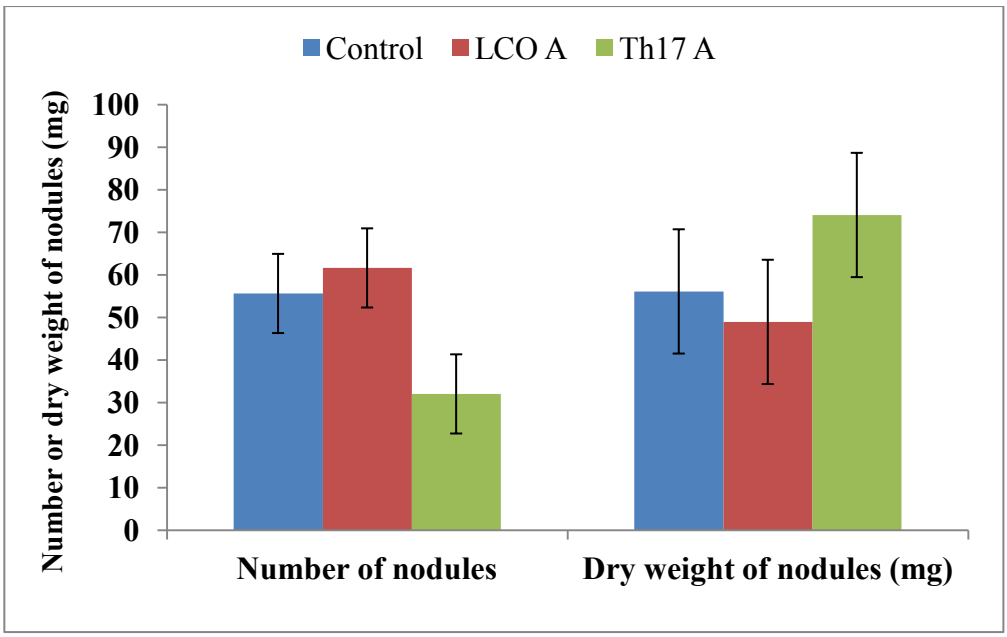

Figure 3 (c)

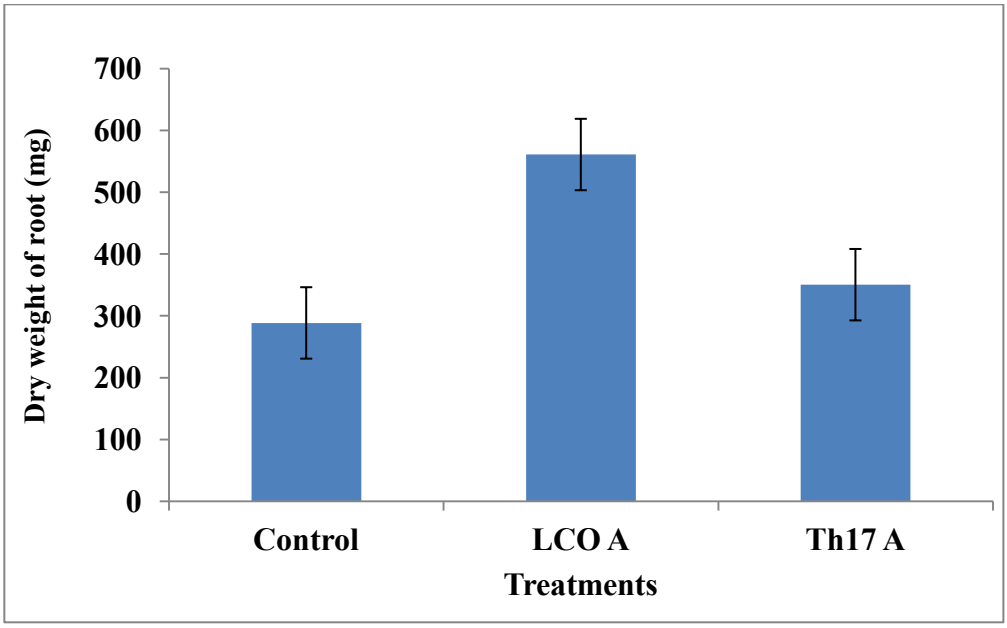

Figure 3 (d)

Connecting statement for Chapter 4

This manuscript was prepared by me, Dr. Donald Smith, Dr. Alfred Souleimanov, Dr. Inna Teshler and Ms. France Rochette. The references cited are presented at the end of the thesis while the tables and figures are presented at the end of Chapter 3.

The manuscript describes the positive effects of Th17 and LCO on the yield of potato under both greenhouse and field growing conditions. Results from the field experiments show that the Th17 and LCO treatments can increase the total yield, marketable yield and numbers of marketable tubers. These studies on potato showed that Th17 has the potential to be used as crop growth enhancer.

Of the co-authors: my supervisor Dr. Donald Smith helped me with his valuable suggestions from start to end of the research. He also went through my manuscripts several times to review and edit the written text. Dr. Alfred Souleimanov helped with extraction and purification of LCO and Th17 used in my treatments. Dr. Inna Teshler helped me in establishing the greenhouse and field experiments. Ms. France Rochette, from DuPont Canada, provided valuable suggestions while I was running my experiments and also provided the data from potato field trials in Ontario.

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Chapter 4

THURICIN17 AND LIPO-CHITOOLIGOSACCHARIDES TREATMENTS ENHANCE POTATO TUBER YIELD

4.1 Abstract

Thuricin 17 (Th17) is a small protein with bacteriocin activity; it is produced by the rhizobacterium *Bacillus thuringiensis* NEB17, which has been shown to improve the growth of crop plants. Lipo-chitooligosaccharides (LCOs), are also produced by rhizobacteria (rhizobia), have plant growth promoting properties similar to Th17 and are now used in commercial crop cultivation. Thus, Th17 was compared with LCO application, in an effort to understand the potential of commercial application of Th17 as a crop growth enhancer. Studies were conducted on potato under greenhouse and field conditions to determine if Th17 can enhance the growth of potato. Data were collected on total yield, marketable yield and number of tubers below market size, market size and above market size. Both Th17 and LCO treatments had positive effects on total and marketable tuber yield under greenhouse and two years of field conditions. Thus, these studies show the potential of Th17 and the need to expand our understanding of the relatively recent and unexpected finding that both Th17 and LCO can act as crop growth enhancers for potato.

4.2 Introduction

Potato (*Solanum tuberosum L.*) is one of the world's most widely cultivated (grown in more than 160 countries) horticultural crops and the fourth most important food crop, in terms of providing calories to humanity, after rice, wheat and corn in 2010 (FAO, 2012). Potato was domesticated in the area now encompassed by Peru and nearby Andean countries around 4000 years ago and was later introduced into Europe, between 1565 and 1580, and brought to USA in 1621 (Acquaah, 2007). In the beginning it was used as food only in monasteries, hospitals and palaces, as it is rich in nutrient content, but with the start of 18th century it became popular among the broader population (Elzebroek and Wind, 2008). Although originally it was grown only under short day conditions of tropical highlands, it can be grown under a wide range of growing conditions, from sea level to an altitude of more than 4000 m (Harris, 1992). After the North American Free Trade Agreement (NAFTA), potato production expanded in Canada (USDA, 2002). Currently, potato is the fifth most widely cultivated agricultural crop in Canada, after wheat, canola, corn and barley. In

addition, Canada ranks 14th in the world for potato production, having produced some 4421770 MT on 139905 ha of land in 2010 (FAO, 2012).

Lipo-chitooligosaccharides (LCOs) are the nod factors produced by the rhizobia (eg. *Bradyrhizobium japonicum*, *Rhizobium*, *Azorhizobium*, *Allorhizobium*, *Mesorhizobium* and *Sinorhizobium*) to signal legume symbiotic partners; they are also able to stimulate plant growth directly (Souleimanov *et al.*, 2002; Prithiviraj *et al.*, 2003; Jose Almaraz *et al.*, 2007). Nod factors have shown various kinds and degrees of influence on growth and development of wide range of crops; stimulation of root growth in *Medicago truncatula* (Oláh *et al.*, 2005); accelerated flowering and increased yield in tomato (Chen *et al.*, 2007); enhanced germination and seedling growth in soybean (Prithiviraj *et al.*, 2003) and increase in photosynthetic rate when applied to soybean leaves (Jose Almaraz *et al.*, 2007). Although it was not clear that LCOs were the causative agent, an LCO containing solution was found to accelerate breaking of potato dormancy in minitubers and microtubers (Habib, 1999).

Th17 is a small protein with bacteriocin activity. It is produced by *B. thuringiensis* NEB17, which was originally isolated from soybean roots (Bai *et al.*, 2002). Bacteriocins are bacteria-produced peptides which kill bacteria closely related to the producer strain (Jack *et al.*, 1995), thus providing a competitive advantage for the producer strain (Wilson *et al.*, 1998). However, it had been shown that the application of Th17 to either leaves or roots of soybean can enhance early seedling growth, photosynthetic rate, soybean nodule number and total fixed N (Lee *et al.*, 2009). As it is a new compound more careful and extensive studies should address the mode of action of plant growth enhancement, range of crops affected, and their specific effects on crop physiology and development. This bacteriocin had also been seen to increase shoot growth in potato (unpublished data). No other studies have been conducted to determine the effect of LCO and Th17 on the growth and yield of potato. Thus the main objective of this study is to examine the effect of Th17 and LCO on potato yield.

4.3 Materials and methods

4.3.1 Preparation of Lipo-chitooligosaccharide

In order to produce and extract LCO, *B. japonicum* strain 532C were cultured in either Yeast Extract Mannitol (YEM) medium under continuous shaking at 150 rpm and a temperature of 27 ± 2 °C. When the OD_{620} reached 0.4–0.5 (4–5 days), genistein (Sigma Chemical Co. MO, USA) was added to a final concentration of 5 μM and the culture was incubated for an additional 48 h. The culture was then extracted with 0.4 vol. of HPLC-grade 1-butanol (Fisher Scientific Canada) by shaking vigorously for 10–15 min and then allowing the material to stand overnight. The organic fraction was then separated and completely dried under vacuum, in a rotary evaporator (Yamato, NJ, USA). The resulting material was dissolved in 18% aqueous acetonitrile and this constituted the LCO extract that was fractionated by HPLC (Fisher Scientific, Montreal, Canada). From time-to-time the chemical identity of the LCO was confirmed by FAB–mass spectroscopy and MALDI–TOF mass spectroscopy (as described by Soulemanov *et al.,* 2002).

4.3.2 Preparation of Thuricin 17

B. thuringiensis NEB17 was cultured in King's Medium B (Atlas, 1995). The initial culture was grown in 250 mL flask containing 50 mL of King's B medium at 25 ± 2 °C. The initial culture was then shaken at 150 rpm on an orbital shaker (Model 5430 Table Top Orbital Shaker) for 48 h. A subculture was prepared from the initial culture by adding 5 mL of the initial culture to 2 L of medium and incubating for a further 48 h under the same growing condition as the initial culture. The bacterial population of *B. thuringiensis* NEB17 was measured spectrophotometrically using as Ultrospec 4300 Pro UV/Visible Spectrophotometer (Biochrom Ltd, Cambridge, UK) at 600 nm (Dashti *et al.*, 1997) and cell-free supernatant containing Th17 was prepared by centrifuging the culture at 13000 g for 10 minutes. Th17 was isolated from these supernatant using HPLC analytical techniques (as described by Gray *et al.*, 2006). The resulting Th17 was purified several times using HPLC until single HPLC peak was isolated.

4.3.3 Greenhouse experiment

One set of experiments was conducted under greenhouse conditions at the Macdonald Campus, McGill University, to determine the effects of Th17 and LCO on potato tuber formation and yield. The treatments consisted of one concentration of Th17 (10^{-10} M) and one of LCO (10^{-7} M), plus a water control. The experiment was organized following a completely randomized design and each treatment was replicated 5 times. The final growth conditions were 22 ± 2 °C, 75% relative humidity with a 16 h photoperiod. However, the temperature was initially set at 18 °C and when the potato shoots started to emerge the temperature was increased gradually (increase 1 °C per day) to 22 ± 2 °C.

Initially, potato tubers (*Solanum tuberosum L.*, variety Norland) were selected based on uniformity of size and uniformity of "eye" (bud) number. The tubers were soaked in the treatment solutions for about 25 min and then were coated with Maxim MZ Potato Seed Protectane (Syngenta Crop Protection Canada, Ontario). Maxim was used at the rate of 500 g (100 kg potato tuber)$^{-1}$ as recommended by the manufacturer. Control tubers were soaked in just water for 25 minutes and then treated with Maxim, in the same fashion as the other treatments. Tubers were sown in 13 L pots. Each pot was filled to the same level with agro-mix and was watered before sowing the tubers. One tuber was sown in each pot, at a depth of 10 cm. After establishing the experiment, the pots were watered every two days until the potato plants were 5 cm tall, after which they were watered daily with equal amounts of water.

Beginning when the potato plants were 15 cm high (36 days after sowing tuber), plants were fertilized weekly with a 20:8.8:16.6 NPK preparation (Plant Products Co. Ltd, Brampton, Ontario, Canada), which also contains traces of boron, cobalt, iron, manganese, molybdenum and EDTA, at the rate of 150 g per 100 L of solution. This fertilization regime was continued until two weeks before harvesting the tubers. Data were collected on time to emergence of shoots.

All tubers were harvested when the plants reached the harvest stage. Before harvesting the tubers, the shoots were harvested and placed separately in paper bags which were placed in a dryer until they reached a constant weight (55 °C for 2 days).

The harvested tubers were divided into groups based on marketable size tubers (3-6 cm diameter), below marketable size tubers (below 3 cm diameter) and above marketable size tubers (> 6 cm diameter). To separate the marketable and unmarketable size tubers 3 and 6 cm diameter size rings were prepared. Those tubers that passed through the 3 cm ring, but did not pass through the 6 cm ring were considered as marketable and the rest as unmarketable. Data were collected on total weight, total number of tubers in each pot, number and weight of marketable size tubers and number of tubers below and above marketable size.

4.3.4 Field trials

4.3.4.1 Field trial at Montreal:

One field study was conducted at the E.A. Lods Agronomy Research Center of the Macdonald Campus of McGill University, Ste-Anne-de-Bellevue, Quebec, Canada from June to September, 2011. The soil at this site has a fine sandy-loam texture. The site had been fallowed for two years before planting potato in 2011.

The experiment was structured to follow a Randomized Complete Block Design (RCBD) with four blocks and five treatments in each block. Each treatment was replicated four times. Each plot consisted of one row of potato plants with 13 plants in each row. Single row plots are representative of field conditions as potato is often produced commercially at a 1 m spacing, to facilitate mechanical weeding; at this spacing the leaves generally do not touch by the end of the growing season, so that the rows do not interact. The inter-row spacing was 1 m and potato tubers were planted at 30 cm between plants within a row. Five treatments were applied: two concentrations of Th7 (10^{-9} and 10^{-10} M) and LCO (10^{-6} and 10^{-7} M) and water only as a control.

The field was plowed to a depth of 20 cm to provide a good bed for planting the tubers. The site was harrowed before sowing the tubers and furrows were opened to sow the tubers into. Tubers were sown manually on June 8th, 2011. Prior to seeding, the tubers were soaked in treatment solution for 25 minutes, in separate plastic buckets. After application of the treatments all tubers were coated with Maxim MZ Potato Seed Protectane (Syngenta Crop Protection Canada, Ontario) applied at 5 g kg^{-1} of potato tubers.

Treated tubers were sown in prepared furrows at a depth of 12-15 cm and covered using a garden hoe. Data were collected on time to emergence, and also on final percentage emergence. When the plants were 15-20 cm tall, fertilizers were applied at the rate of 100:21.8:41.5 NPK kg ha^{-1}. Nitrogen, phosphorous and potassium were supplied using diammonium phosphate (18-46-0 NPK), urea (46-0-0 NPK) and muriate of potash (0-0-52 NPK). After applying fertilizers hilling (15-20 cm high) was performed by bringing the soil up around the stems of the potato plants.

The tubers were harvested when all the plants reached the harvest maturity stage. At this stage the plant loses its leaves, tuber growth ceases, the shoots turn yellow and then die. Data was collected from 10 randomly selected plants in each plot. First and last plants within the row were excluded to decrease border effect. As in the greenhouse experiment, all the tubers harvested from each plant were grouped into marketable size (3-6 cm diameter), below marketable size (> 3 cm diameter) and above marketable size (> 6 cm diameter) ranges. Data were collected on total yield, total number of tubers, weight and number of marketable size tubers, weight of tubers greater and lower than marketable weight and number of smaller than marketable size tubers per plant.

4.3.4.2 Field trial in Ontario:

In addition to the field trial at the Macdonald Campus, another field trial was conducted in 2010 at Walking Field, Thorndale, Middlesex, Ontario from June to September. The field had a medium loam texture with 41 % sand, 39 % silt and 20 % clay. Corn was grown on the field in the previouse year. The experiment was organised following a Randomized Complete Block Design with four blocks and four replications of each treatment. Each plot consisted of 2 rows of potato plants and measured 2.5 m wide, 8.0 m long and had an area of 20 sq. m. The inter-row spacing was 1 m and the potato tubers were planted at a 40 cm spacing within each row. The potato varietiy used was Shepody in 2010.

Three concentrations of Th17 (10^{-9}, 10^{-10} and 10^{-11} M) and LCO (10^{-6}, 10^{-7} and 10^{-8} M) were utilized. Water treatment was used as control. The tubers were planted manually at the depth of 10 cm on June 8th, 2010 and 2011. The treatments were

applied to potato seed pieces prior to seeding, followed by dusting with Maxim MZ Potato Seed Protectane (Syngenta Crop Protection Canada, Ontario) applied at the rate of 5 g kg^{-1} tubers. The treated seed pieces were planted within 24 h of treatment. Recommended rates of fertilisers were broadcast during seed bed preparation, at the rate of 120:26.2:49.8 NPK kg ha^{-1}.

Data were collected when the potato tubers reached the harvest stage. During the season data were collected on emergence and time of flowering. At harvest maturity one row from each plot (20 plants from one plot) was harvested and data were collected on biomass of the shoots, total yield, marketable yield, and unmarketable yield. Unlike the trial at the McGill site, in this trial, the total yield was divided into only two groups: marketable and unmarketable yield. The unmarketable yield consisted of tubers below 0.55 cm in diameter plus misshapen or "knobby" potatoes; and the marketable yield was calculated as the difference between total and unmarketable yield.

4.3.5 Data analysis:

Data were analyzed using PROC MIXED (SAS Institute 2009) for the field trials in Montreal based on a Completely Randomized Block design while PROC GLM was used for the greenhouse experiments following a Completely Randomized Design. For the greenhouse study, treatment means were compared using least significant difference (LSD) at $P < 0.05$ while treatment means from the field experiments in Montreal were compared using a Scheffe's multiple comparisons test at the 95% confidence interval ($P < 0.05$). Data from the Ontario trial were analyzed using PROC GLM (SAS Institute 2009) and treatment means were compared using a protected least significant difference (LSD) test at $P < 0.05$.

The significance of the treatments was determined at the 5% probability level, or at 95 % confidence level. However, where biologically or agronomically interesting effects occurred at the 10% probability level these are reported and the P values are given.

4.4 Results

4.4.1 Effect of Th17 and LCO on total and marketable yield under controlled environment condition in green house

The results showed that there was no effect of treatments on total number of tubers, total yield or marketable yield; although the marketable yield from LCO and Th17 treatments were numerically greater (Table 8). In addition, Th17 and LCO treatments resulted in numerically fewer unmarketable tubers than the control treatment. Both marketable and unmarketable yields were converted to percentage of total yield. These values showed that both LCO and Th17 treatments resulted in higher percentages of marketable yield and lower unmarketable yields, as compared to water control (Figure 4 (a)).

4.4.2 Effect of Th17 and LCO on total and marketable yield under field conditions in Montreal

Based on the results from the greenhouse study, we conducted field trials, to determine the effect of LCO and Th17 on potato yield under conditions closer to those used by commercial producers. The results showed clearly that both LCO and Th17 treatment increased the marketable weight of potato. Th17 (10^{-9} M) increased the total number of tubers ($P < 0.053$) per plant and the number of tubers between 3-6 cm size ($P < 0.065$) per plant (Table 10). Although both LCO and Th17 treatments increased the marketable size tuber ($P < 0.086$), they did not affect total yield. However, for some treatments LCO (10^{-7} M), Th17 (10^{-9} M) and Th17 (10^{-10} M) total yield was increased numerically by 11, 13 and 12 % respectively (Figure 5 (b)). The weight of tubers between 3-6 cm was increased ($P < 0.08$) by 8.7 and 27% respectively by LCO (10^{-6} M) and LCO (10^{-7} M) while Th17 (10^{-9} and 10^{-10} M) increased the marketable yield ($P < 0.08$) by 39.7 and 21.3%, respectively, as compared to the water control treatment.

4.4.3 Effect of Th17 and LCO on total and marketable yield under field conditions in Ontario

The results from the 2010 Ontario trial showed clearly that all three concentrations of both LCO and Th17 treatment increased the total yield, marketable yield and marketable numbers of the potato tubers. The ANOVA showed that all the three

concentrations of LCO (10^{-6}, 10^{-7} and 10^{-8} M) and Th17 (10^{-9}, 10^{-10} and 10^{-11} M) increased the total yield ($P < 0.0001$), marketable yield ($P < 0.0001$) and numbers of marketable tubers ($P < 0.0002$). However, unmarketable yield was not affected by the use of LCO and Th17 treatments ($P < 0.507$) (Table 9). LCO (10^{-6}, 10^{-7} and 10^{-8} M) increased total yield by 110, 106 and 134 %, respectively, while Th17 (10^{-9}, 10^{-10} and 10^{-11} M) increased total yield by 137, 167 and 194 %, respectively, over the control treatment. Similarly LCO (10^{-6}, 10^{-7} and 10^{-8} M) increased the marketable yield by 110, 104 and 135 %, respectively, and Th17 (10^{-9}, $10^{-10}$and 10^{-11} M) increased the marketable yield by 139, 172 and 199 %, respectively, as compared to water control (Figure 6 (b)).

4.5 Discussion

This work provides the first reported data regarding the effects of Th17 on potato yield. Before this work, LCO and Th17 had been assessed for growth promoting effects on soybean, corn, barley, tomato and several other crops, but for potato only one study had previously been conducted, to assess the effect of an LCO containing material on breaking the dormancy of cold stored micro- and mini-potato tubers. It was reported that when micro-tubers were soaked in signal solution for 24 h and incubated for one week, it can induce more sprouts than are formed on water soaked control micro-tubers (Habib, 1999).

Based on both greenhouse and field experiments, it had been shown that both LCO and Th17 had positive effects on the marketable tuber yield. From the greenhouse work we were not able to see statistically significant differences for the measured variables, but there were numerical increases due to treatment with LCO and Th17, that increased the % of marketable size tubers by 7.16 and 8.17%, respectively, as compared to water control (Figure 4 (b)). All the data presented from the greenhouse work are from an average of 5 plants, given on a per plant basis. A 7 to 8 % increase per plant is important in terms of commercial cultivation given the low concentrations of signal compounds required and, therefore, the potential low cost of these treatments in a commercial context. These findings have the potential to have been economically significant. This work has been shown that Th17 treatment can increase the yield in

potato; the cost of production of Th17 is likely to be low, because of the very low concentrations generally required (10^{-9} to 10^{-11} M). For many crops the use of Optimize, an LCO containing product, has already been shown to be economically viable. However, TH17 should be easier to produce and the bacterium that produces it grows substantially faster than the rhizobia, and Th17 is produced in larger amounts. In addition, Th17 is effective at much lower concentrations than LCO. Thus, it seems very likely that the use of Th17 as a commercial technology will be less expensive than LCOs, which are already established in the market place.

The % marketable yield and unmarketable yield out of total yield showed that both LCO and Th17 treatments resulted in higher % marketable tuber size and less % unmarketable yield, as compared to the water control. From this we could also say that LCO and Th17 treatments helped to decrease the proportion of unmarketable size tubers and increase the proportion of marketable size tubers.

There a number of factors that affects the quality and quantity of tuber formation in potato. One of the factors that might have affected these variables in our greenhouse study was growing the plants in pots. Various studies had been conducted to determine the difference between growing potatoes under controlled environment (in pots) and field conditions. Potato grown in beds had greater weights and longer shoots than potatoes grown in pots in a greenhouse. Here plastic pots could create physical constraints to good tuber production. Based on these circumstances and the positive increase in marketable size tubers; we repeated the testing in the field from June to September, 2010 and 2011.

The results from field were as we had expected based on greenhouse results. The treatments resulted in increases in total number of tubers per plant, total number of marketable tubers per plant and marketable yield per plant in both years of field trials (Table 9 and 10). However, under field conditions these increases were larger and achieved statistical significance. At the same time, it should be noted that there were no changes in total yield per plant due to signal compound treatment under field conditions in 2011, rather the important changes were in mass of marketable tubers. In the 2010 trial there was an increase in total yield due to Th17 treatment. Among the

evaluated treatments, Th17 (10^{-9} M) resulted in higher total yield, the highest marketable yield and the least yield for the tubers that were bigger that 6 cm in diameter in 2011 trial. For potato, from a market prospective, tubers that are excessively large or small are not salable.

In the 2010 field trial, the total yield was only separated in two groups: marketable and unmarketable yield. Tubers that were smaller than 0.55 cm in diameter were considered unmarketable and above 0.55 cm were marketable yield. LCO (10^{-6}, 10^{-7} and 10^{-8} M) increased the marketable yield by 110, 104 and 135 %, respectively, and Th17 (10^{-9}, $10^{-10}$and 10^{-11} M) increased the marketable yield by 139, 172 and 199 %, respectively, as compared to the water control. Here the marketable yield was increased by more than 100 % and Th17 (10^{-11} M) increased the marketable yield by almost 200 %, over the water control (Figure 6 (b)). This increase in yield is very important from commercial point of view.

In 2011 trial, the total tuber yield from each plant was separated in three groups: less than 3 cm diameter which are below marketable size; 3-6 cm diameter which are desirable and marketable; and more than 6 cm diameters, which are also less desirable marketable yield, because of their larger size. LCO (10^{-6} and 10^{-7} M), Th17 (10^{-9} and 10^{-10} M) increased the total yield by 1.3, 11.6, 13.5 and 12.3 %, respectively, over the water-only controls (Figure 5 (b)). In the field of 2011, the total yield was numerically higher than water control. An almost 10% increase in total yield can mean a lot for commercial potato producers. Similarly, LCO (10^{-6} and 10^{-7} M), Th17 (10^{-9} and 10^{-10} M) increased the marketable size tubers (3-6 cm diameter) by 8.7, 27, 39.7 and 21.3 %, respectively, over the water control. Among all the treatments Th17 (10^{-9} M) resulted in the highest total yield, marketable yield, total tubers and total marketable tubers.

These results generally support previous findings from our laboratory; however the results were for each of the two crops tested, corn and soybean. When Th17 was applied to either leaves or roots of soybean plant, they increase the leaf area, plant dry matter accumulation and photosynthetic rate (Lee *et al.*, 2009). Similarly, LCO treatment also increased leaf area, root and shoot length, and root and shoot dry matter in soybean plant (Prithiviraj *et al.*, 2003). In both of these experiments LCO or Th17

were chronically provided to the plant. However in our experiment with potato, tubers were exposed only once, an acute treatment. My findings are generally consistent with those of Lee *et al*. (2009) and Prithiviraj *et al*. (2003) but add some additional perspective regarding interactions with stress in the case of soybean.

The increase in germination, leaf area, dry matter accumulation in roots or pods, could be because of hormone like effects of LCOs (Prithiviraj *et al.*, 2003). LCO spray on tomato accelerates flowering (a typical response to stress), and increases yield (Chen *et al*. 2007); LCO increases root growth of Arabidopsis (Khan *et al.*, 2011); Nod factors can induce the cell division (Schlaman *et al.*, 1997) not only in legumes but also in non-legumes (De Jong *et al.*, 1993; Dyachok *et al.*, 2000); and also stimulate early somatic embryo development in Norway spruce (Dyachok *et al.*, 2002). Enhanced germination and seedling growth, along with the mitogenic nature of LCOs, suggest accelerated meristem activity. This may lead to increased sink demand and the observed increases in mobilization of seed reserves (Prithiviraj *et al.*, 2003) and increased photosynthetic rates (Jose Almaraz *et al.*, 2007) for more developed plants; both of which lead to increased growth (Khan *et al.*, 2008). For potato, high yield could be achieved via higher photosynthetic rates, if other factors are kept the same (Dwelle, 1985), which could be the case in our experiments LCOs are also known to activate specific plant genes and act as mitogens (Long, 1989; Dénarié *et al.*, 1996; Perret *et al.*, 2000; Patriarca *et al.*, 2004).

Potato is a cool weather loving plant, and is very sensitive to high temperature, especially tuber formation. The optimum temperature for potato tuber formation is around 18 °C. With increasing temperature the dry matter accumulation in plants and tubers are decreased (Lafta and Lorenzen, 1995). Potato cultivation in Canada is generally started in early spring when the soil temperature goes above 10 °C. In our studies, the greenhouse experiment was started at around 18 °C and the temperature was increase slowly to 22 °C, after the emergence of shoots, and was maintained at 22 °C for the rest of growing period. However, the temperature was close to or above 25 °C when we started our field trial on 8th June, 2010 and 2011 (Figure 8 (a)). This indicated that the field grown potato plants were probably stressed and the growing

temperature was higher than optimal during tuber production. However, we were still able to harvest a good amount of tubers from our plants. This good harvest, as we hypothsize, could be because of the signal treatments as the treatments helped the potato plant to overcome the high temperature stress. These results are consistent with the results from our soybean studies in which LCO and Th17 treatments helps the soybean seeds to overcome the low temperature stress conditions and maintain the good emergence under low temperature stress.

4.6 Conclusions

My studies were conducted on potato under greenhouse and field conditions and showed that both LCO and Th17 could influence the yield of potato plants; both total and marketable yield. This was the first study to show the positive effects of LCO and Th17 on potato growth and yield. Between LCO and Th17, Th17 performed better with regard to yield of potato plants. Th17 is a newer compound than LCO, which is already in the market place. Thus, we compared the Th17 to LCO in our studies to know the potential of Th17 in the marketplace. These studies on potato showed that Th17 has potential to be used as crop growth enhancer. However, there is still a lot of characterization and research required, in order to better understand the mode of action and range of crops being influenced.

Table 8: ANOVA and LSD test for the effects of Th17 and LCO on total and marketable yield of potato under greenhouse conditions. The t groupings obtained from LSD analysis are given only for those variables which were significantly different (at $P < 0.05$). Means associated with the same letter are not different by the ANOVA protected LSD test. (10-7 and 10-10 M indicate molar concentration of either LCO or Th17)

Parameter	**ANOVA**		**LSD for comparison among treatments**	
	Source	**P value**	**Treatment**	**Means ± SE**
Total Numbers of Tubers	Treatment	0.338^{NS}	Control	7.6 ± 1.2
			LCO (10^{-7})	5.4 ± 1.2
			Th17 (10^{-10})	7.6 ± 1.2
Total Yield per plant (gm)	Treatment	0.798^{NS}	Control	461.06 ± 28.9
			LCO (10^{-7})	434.04 ± 28.9
			Th17 (10^{-10})	452.62 ± 28.9
Marketable yield per Plant (gm)	Treatment	0.274^{NS}	Control	373.58 ± 25.0
			LCO (10^{-7})	400.32 ± 25.0
			Th17 (10^{-10})	404.1 ± 25.0
Unproductive Yield per plant (gm)	Treatment	0.201^{NS}	Control	87.48 ± 20.5
			LCO (10^{-7})	33.72 ± 20.5
			Th17 (10^{-10})	48.52 ± 20.5

* indicates significant differences at the 0.1 probability level and ^{NS} indicates no significant difference. SE = standard error of the mean. Means presented in the above table are the average of 5 replicates.

Table 9. ANOVA and LSD test for the effect of Th17 and LCO on total and marketable yield of potato under field conditions at Walking field, Thorndale, Middlesex, Ontario, Canada in 2010. The t groupings obtained from LSD analysis are given only for those variables which were significantly different (at $P < 0.05$). Means associated with the same letter are not different by the ANOVA protected LSD test. (10^{-6}, 10^{-7}, 10^{-8} 10^{-9}, 10^{-10} and 10^{-11} indicate molar concentrations of either LCO or Th17)

Parameter	**ANOVA**		**LSD for comparison among treatments**		
	Source	P value	Treatment	Means ± SE	t grouping
Total Yield (kg)	Treatment	< .0001***	Control	7.8 ± 1.3	D
			LCO(10^{-6})	16.38 ± 1.3	C
			LCO (10^{-7})	16.07 ± 1.3	C
			LCO (10^{-8})	18.27 ± 1.3	BC
			Th17 (10^{-9})	18.48 ± 1.3	BC
			Th17 (10^{-10})	20.85 ± 1.3	AB
			Th17 (10^{-11})	22.94 ± 1.3	A
Number of Marketable Tubers	Treatment	0.0002***	Control	50.6 ± 10.5	C
			LCO(10^{-6})	121.3 ± 10.5	AB
			LCO (10^{-7})	103.3 ± 10.5	B
			LCO (10^{-8})	135.3 ± 10.5	AB
			Th17 (10^{-9})	126.6 ± 10.5	AB
			Th17 (10^{-10})	139 ± 10.5	A
			Th17 (10^{-11})	150.3 ± 10.5	A
Mark Yield (kg)	Treatment	< .0001***	Control	7.42 ± 1.2	D
			LCO(10^{-6})	15.59 ± 1.2	C
			LCO (10^{-7})	15.1 ± 1.2	C
			LCO (10^{-8})	17.47 ± 1.2	BC
			Th17 (10^{-9})	17.72 ± 1.2	BC
			Th17 (10^{-10})	20.17 ± 1.2	AB
			Th17 (10^{-11})	22.16 ± 1.2	A

Parameter	ANOVA		LSD for comparison among treatments		
	Source	P value	Treatment	Means ± SE	t grouping
Unmarketable Yield (kg)	Treatment	0.507NS	Control	0.41 ± 0.17	
			LCO(10^{-6})	0.78 ± 0.17	
			LCO (10^{-7})	0.95 ± 0.17	
			LCO (10^{-8})	0.79 ± 0.17	
			Th17 (10^{-9})	0.76 ± 0.17	
			Th17 (10^{-10})	0.68 ± 0.17	
			Th17 (10^{-11})	0.78 ± 0.17	

***indicates high significant differences at the 0.01 probability level and ^{NS} indicates no significant differences at the 0.1 probability level. SE = standard error of the mean. Means presented in the above table are the average of all the plants from one plot (20 plants from one plot).

Table 10. ANOVA and LSD test for the effect of Th17 and LCO on total and marketable yield of potato under field conditions (Montreal 2011). The t groupings obtained from LSD analysis are given only for those variables which were significantly different (at $P < 0.05$). Means associated with the same letter are not different by the ANOVA protected LSD test. (10^{-6}, 10^{-7}, 10^{-9} and 10^{-10} indicate molar concentrations of either LCO or Th17.)

Parameter	**ANOVA**		**LSD for comparison among treatments**		
	Source	P value	Treatment	Means ± SE	t grouping
Total Number of Tubers	Treatment	0.050**	Control	6.06 ± 0.3	B
			LCO (10^{-6})	5.76 ± 0.3	B
			LCO (10^{-7})	5.9 ± 0.3	B
			Th17 (10^{-9})	7.06 ± 0.3	A
			Th17 (10^{-10})	6.13 ± 0.3	B
Number of Tubers between 3-6 cm	Treatment	0.065*	Control	3.76 ± 0.32	B
			LCO (10^{-6})	3.66 ± 0.32	B
			LCO (10^{-7})	4.03 ± 0.32	B
			Th17 (10^{-9})	4.9 ± 0.32	A
			Th17 (10^{-10})	4.1 ± 0.32	B
Total Yield (gm)	Treatment	0.319^{NS}	Control	542.68 ± 30.7	
			LCO (10^{-6})	549.84 ± 30.7	
			LCO (10^{-7})	605.73 ± 30.1	
			Th17 (10^{-9})	616.14 ± 30.1	
			Th17 (10^{-10})	609.55 ± 29.8	
Weight of Tubers between 3-6 cm (gm)	Treatment	0.086*	Control	340.29 ± 31.5	C
			LCO (10^{-6})	369.92 ± 31.1	BC
			LCO (10^{-7})	432.47 ± 31.5	AB
			Th17 (10^{-9})	475.35 ± 31.5	A
			Th17 (10^{-10})	412.91 ± 31.5	B

**indicates significant differences at the 0.05 probability level; * indicates significant differences at the 0.1 probability level and ^{NS} indicates no significant differences at 0.1 probability level. SE = standard error of the mean. Means presented in the above table are the average of 40 replicates.

Figure 4(a): The effect of LCO or Th17 on potato yield under greenhouse conditions. The figure shows the proportion of the marketable yield and unmarketable yield for each treatment. Here unmarketable yield is the difference between total and marketable yield. Each bar graph shows the proportion of marketable and unmarketable yield out of total yield for the particular treatment. Each point in the graph is the average of 5 replicates. LCOA: LCO (10^{-7} M), Th17A: Thu17 (10^{-10} M) and Control: water control.

Figure 4(b): The effect of LCO or Th17 on potato yield under greenhouse conditions. The figure shows the % increase in marketable yield of potato caused by the treatments, as compared to water control. Each bar graph shows the percentage increase in marketable yield, over control, for the particular treatment. Each point on the graph represents the average of 5 replicates. LCOA: LCO (10^{-7} M), Th17A: Th17 (10^{-10} M) and Control: water control.

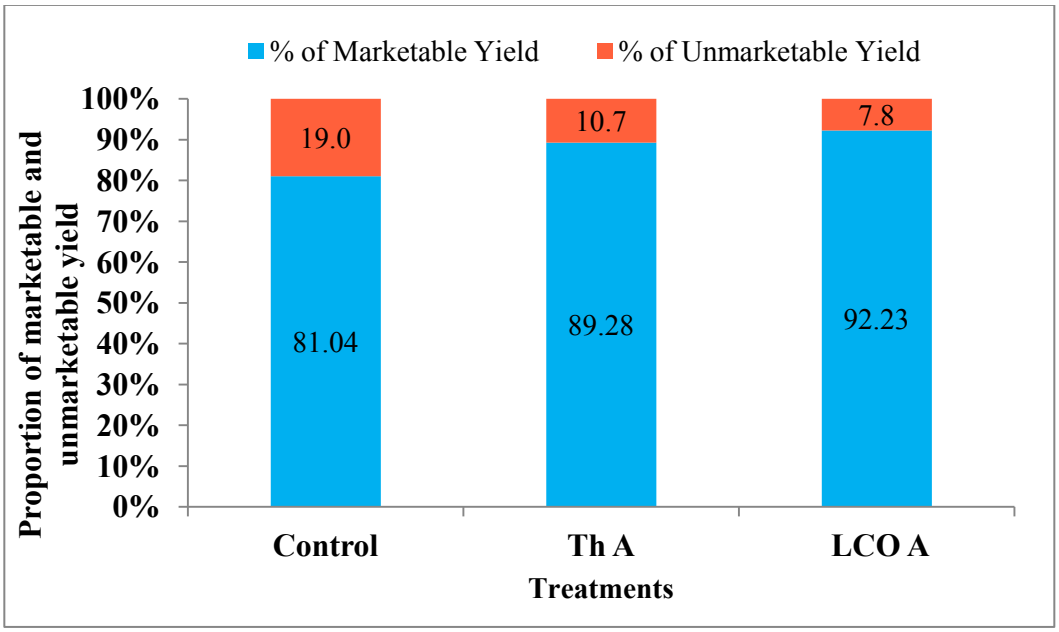


Figure 4 (a)

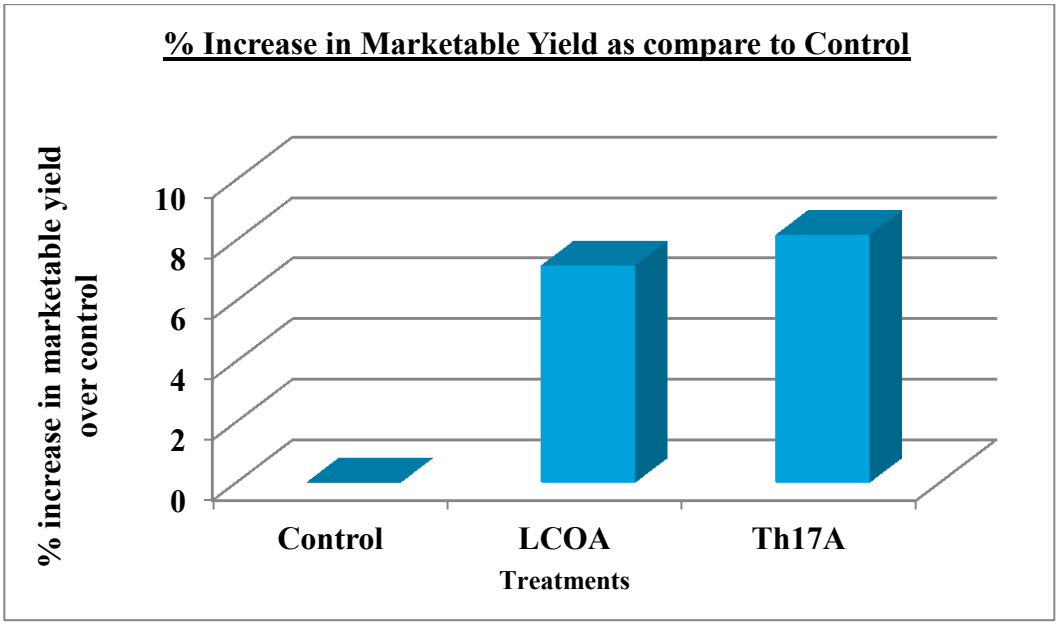


Figure 4 (b)

Figure 5(a): The effect of LCO or Th17 on the yield of potato (Montreal 2011). The figure shows the yields of the three tuber size classifications: tubers between 3-6 cm diameter, tubers larger than 6 cm diameter and total yield per plant. Each bar graph in the figure represents the average reading from 40 plants and is given +/- standard error. Weight 3-6 cm: weight of tubers that are > 3 cm and < 6 cm in diameter; Weight > 6cm: weight of tubers that are greater than 6 cm in diameter; LCOA: LCO (10^{-6} M); LCOB: LCO (10^{-7} M); Th17A: Th17 (10^{-9} M); Th17B: Th17 (10^{-10} M) and Control: water control.

Figure 5(b): The effect of LCO and Th17 on % increase in potato yield, as compared to the water control under field condition (Montreal 2011). The figure shows the % increase caused by LCO and Th17 treatments on weight of tubers more that 3 cm diameter, 3-6 cm diameter and total yield as compared to the water control. Each bar graph in the figure is the average reading from 10 plants. LCOA: LCO (10^{-6} M), LCOB: LCO (10^{-7} M), Th17A: Thuricin 17 (10^{-9} M), Th17B: Thuricin 17 (10^{-10} M) and Control: water control.

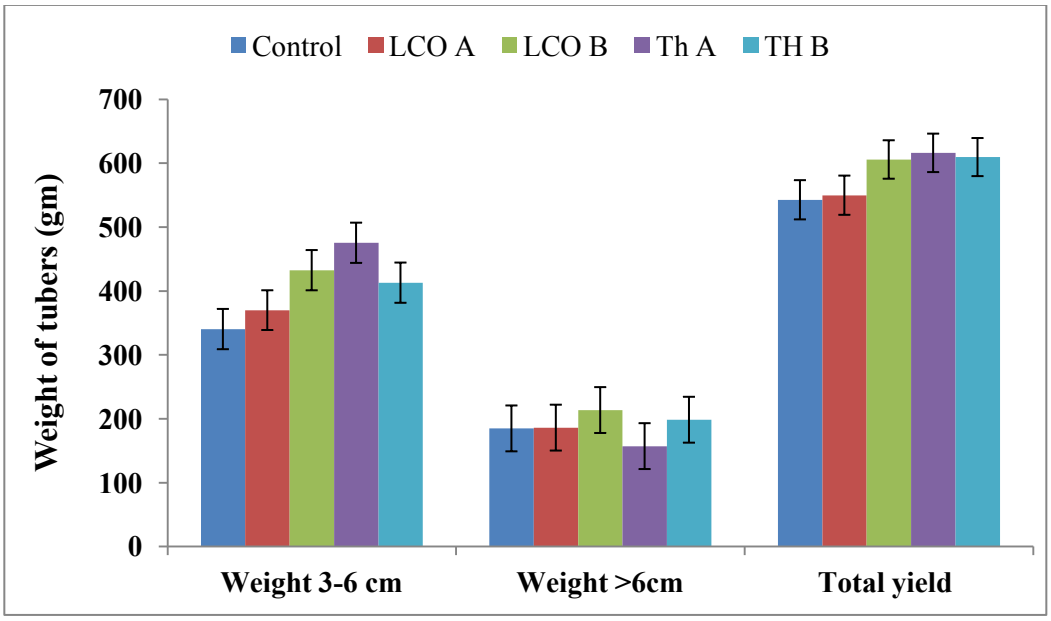


Figure 5 (a)

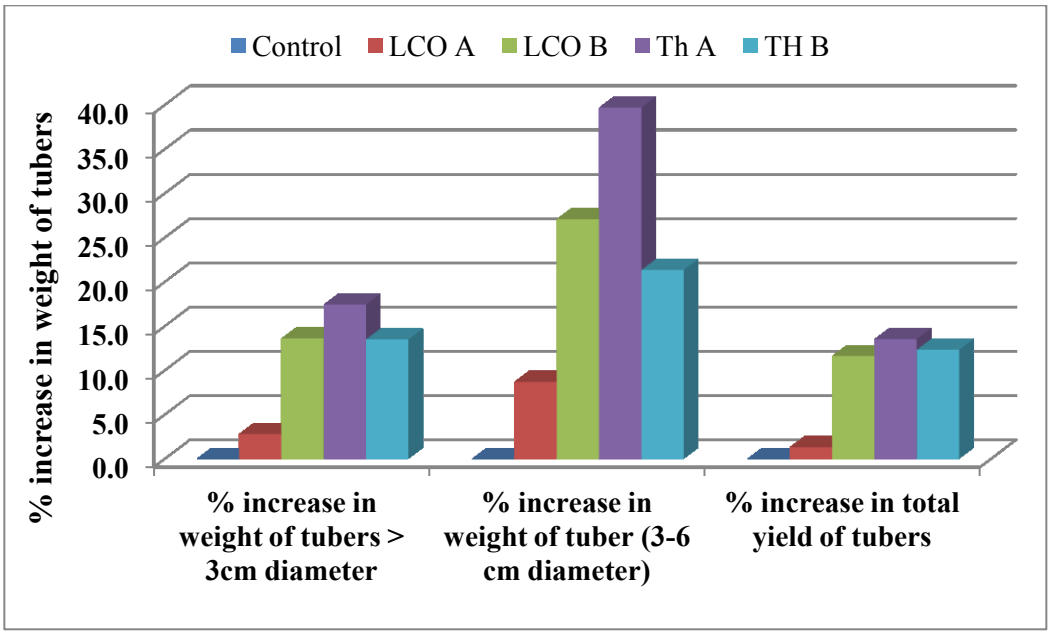


Figure 5 (b)

Figure 6(a): The effect of LCO or Th17 on the yield of potato (Ontario 2010). The figure shows three different yields: unmarketable, marketable and total yield from the field trials in Ontario, 2010. Each bar graph in the figure represents the average reading for one experimental plot (20 plants in each plot) and is given +/- standard error. LCOA: LCO (10^{-6} M), LCOB: LCO (10^{-7} M), LCOC: LCO (10^{-8} M), Th17A: Th17 (10^{-9} M), Th17B: Th17 (10^{-10} M), Th17C: Th17 (10^{-11} M) and Control: water control.

Figure 6(b): The effect of LCO and Th17 on % increase in potato yield (Ontario 2010), as compared to the water control. The figure shows the % increase caused by LCO and Th17 treatments on total and marketable weight as compared to the water control. Each bar graph in the figure represents the average reading for one experimental plot (20 plants in each plot). LCOA: LCO (10^{-6} M), LCOB: LCO (10^{-7} M), LCOC: LCO (10^{-8} M), Th17A: Th17 (10^{-9} M), Th17B: Th17 (10^{-10} M), Th17C: Th17 (10^{-11} M) and Control: water control.

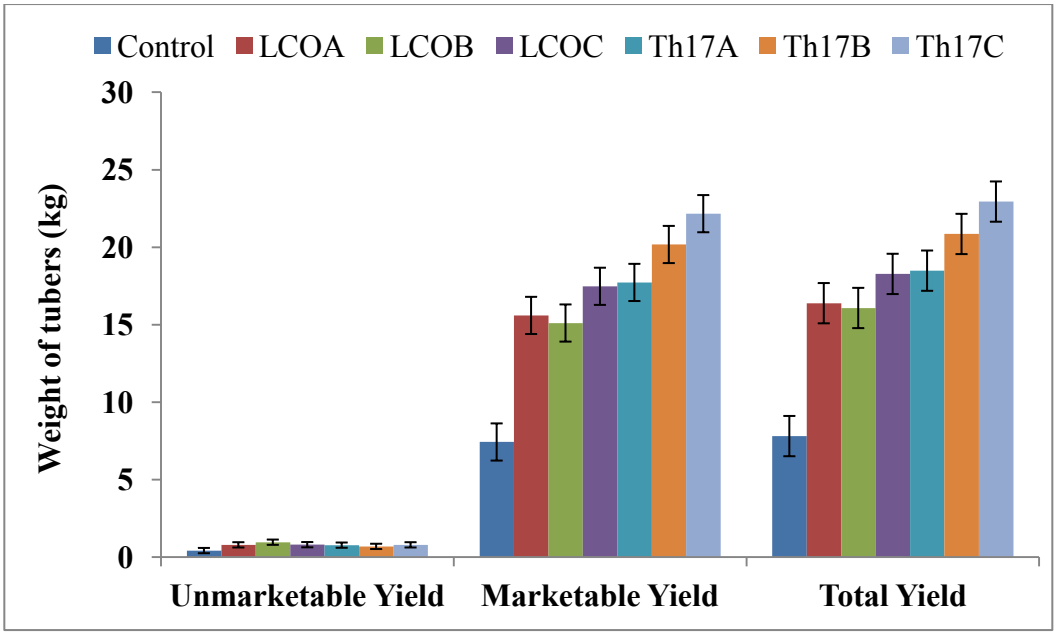


Figure 6 (a)

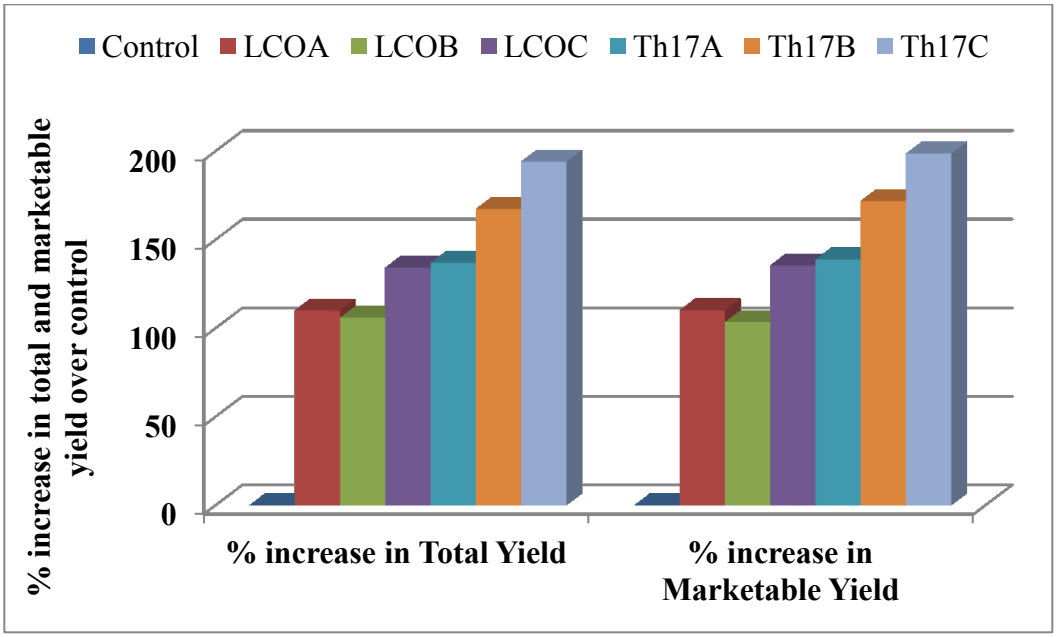


Figure 6 (b)

Chapter 5 GENERAL DISCUSSION; CONCLUSIONS AND ACCEPTANCE OR REJECTION OF HYPOTHESIS

The legume-rhizobia symbiosis is one of best studied and widely researched plant-microbe interactions. This interaction between plants and micro-organisms are facilitated by signal molecules (Walker *et al.*, 2003), which are detected by the interacting partners (Mabood and Smith, 2005). As a result of this interaction, special structures, nodules, are formed on plant roots. Nodulation in legumes is a complex process consisting of various steps, in a clear sequence. In the initial stage of nodulation phenolic compounds, flavanoids – plant-originating signals, are produced and trigger the activation of *nod* genes in rhizobia (Zaat *et al.*, 1987). These *nod* genes cause the production of nod factors, which are lipo-chitooligosaccharides (LCOs) – bacteria-originating signals (Schultze and Kondorosi, 1998). In general, most LCOs have similar structures, consisting of a 3-5 chitin unit backbone (a linear chain of β-1, 4- linked N-acetylglucosamines) linked to an acyl chain (Mergaert *et al.*, 1997), and with various modifications to the reducing and non-reducing ends of the chitin backbone. The LCOs produced by *B. japonicum* (eg. Nod Bj-V C18:1, MeFuc) have a pentameric backbone with C18:1, C16:0 and C16:1 fatty acid chains at the non-reducing end and 2-0-methylfucose at the reducing end of the chitin backbone (Carlson *et al.*, 1993).

The PGPR *B. thuringiensis* NEB17 was isolated from soybean nodules (Bai *et al.*, 2002) and has been shown to increase growth and nodulation of soybean plants when applied as a co-inoculant with *B. japonicum* 532C (Bai *et al.*, 2003). This bacterium produces the bacteriocin Th17, with molecular weight 3.1 kDa, which is not toxic to *B. japonicum* 532C (Gray *et al.*, 2006b). Bacteriocins are bacteria-produced peptides which generally kill bacteria that are closely related to the producer strain (Jack *et al.*, 1995), hence providing a competitive advantage for the producer strain (Wilson *et al.*, 1998). It had been already demonstrated that the application of Th17, to either leaves or roots, can enhance plant growth, early seedling growth, photosynthetic rate, soybean nodule number and total fixed N (Lee *et al.*, 2008). However, a great deal of research remains to be done regarding matters such as the

range of crops affected, interaction with crop stress and their specific effects on crop physiology and development.

5.1 Soybean

In my first study with soybean, I have shown how Th17 and LCO treatments can enhance soybean growth under a range of growing conditions extending from growth chambers to the field. My studies include a series of experiments investigating the effects of chronic (germination assays) and acute (greenhouse and field trials) exposure of Th17 and LCO on soybean growth. The results from germination studies showed clearly that both Th17 and LCO enhanced plant growth directly either at a mildly stressful growing temperature (22 ± 2 °C) or quite severely stress temperature (15 ± 2 °C), and that the compound had more pronounced effects under the more stressful condition. Under greenhouse conditions, acute exposure of the Th17 and LCO treatments resulted in higher dry weight of pods, heavier roots and higher nodule weight per plant than the water control. However, they did not have any effect on leaf area, number of trifoliate leaves, number of pods and total dry weight of the shoots. Similarly, under field conditions in 2010, Th17 and LCO treatment increased the leaf area, total plant biomass, nodule number and nodule biomass over the control treatment. But, we were not able to see any differences from the field trials conducted in 2011, when conditions were less stressful. This is the first time we have evaluated the effects of acute exposure to Th17 and LCO treatments under field conditions.

Soybean is a sub-tropical crop which requires temperatures of 25 – 30 °C for optimum growth. Low temperature not only limits the growth of soybean but also hinders to the initiation of nodulation, nitrogen fixation and finally the yield. Here, I have demonstrated that the use of Th17 helps to overcome low temperature stress effects during the emergence of soybean seedlings, and to maintain good early growth thereafter. However acute exposure of Th17 treatments enhanced growth sometimes, although sometimes we were not able to see significant increases in growth. For acute exposure, the efficacy of the treatments can be enhanced, with the use of various technologies developed for seed treatment. For Canadian producers, this technology is particularly important as it offers an opportunity to improve crop growth under low

temperature conditions, prevalent during the early growing season in Canada, and potentially support crop diversification in early maturity zones.

5.2 Potato

My studies with potato show clearly that LCO and Th17 can influence the yield of potato plants under greenhouse and field conditions. This was the first reported study to show the positive effects of LCO and Th17 on potato growth and yield. The Th17 and LCO treatments resulted in increases in total number of tubers per plant, total number of marketable tubers per plant and marketable yield per plant, and reduced the yield of tubers which are excessively large in size. For potato, tubers that are excessively large or small are not salable.

Potato is a cool weather loving plant, and the optimum temperature for potato tuber formation is around 18 °C. With increasing temperature the dry matter accumulation in plants and tubers are decreased (Lafta and Lorenzen 1995). Potato cultivation in Canada is generally started in early spring when the soil temperature goes above 10 °C. In our studies, the greenhouse experiment was started at around 18 °C and the temperature increased slowly to 22 °C, after the emergence of shoots, and was maintained at 22 °C for the rest of growing period. However, the temperature was close to or above 25 °C when we started our field trial on 8th June, 2011. This indicated that the field grown potato plants were probably stressed in that the growing temperature was higher than optimal during tuber production. However, we were still able to harvest a good amount of tubers from our plants; the treatments may have helped to overcome the high temperature stress. These results are consistent with the results from our soybean studies in which LCO and Th17 treatments helps the soybean seeds to overcome the low temperature stress conditions and maintain the good emergence under low temperature stress.

5.3 Broad effects, across both crops

Overall, we have shown, through both of our studies (soybean and potato), that a very low concentration of our treatments (Th17 and LCO) enhances the growth aspects of both soybean and potato. Potato is a C_3 plant and belongs to Solanaceae family, while soybean is also a C_3 plant it belongs to Fabaceae family; they are very

distantly related. In addition, they evolved under very different conditions: potato under cooler conditions at high altitudes in the Andes Mountains, and soybean in what is now south coastal China, where conditions are warm. As a result they have different optimal climatic growth conditions. For example the optimum thermal growing conditions for soybean are 25 – 30 °C while potato does best at temperature around 18 °C. Temperatures that are optimal for potato can be stressful cool for soybean. Potato is particularly sensitive to higher temperatures during tuber formation. In our studies, we found that we can enhance the growth of both crops, under either stressfully high or low temperature growing conditions through the use of Th17 or LCO treatments.

These finding shows that both of the evaluated compounds have potential to effect the growth of a wide range of crops with a range of physiologies. In addition to these crops, previous work has been conducted on corn, wheat and tomato and demonstrated that these treatments can enhance their growth and development. Overall, the current study shows the potential for commercial application of Th17 as a crop growth enhancer over a wide range of crops.

5.4 General conclusions

With rising energy costs and increased public awareness of environmental issues, there is a need to develop sustainable agricultural inputs, such as crop growth stimulants effective at very low concentrations and integrated with current agronomic practices. This is a serious challenge for the entire agriculture sector and, as we begin to exploit biofuels, the energy sector as well. Here we have shown that very low concentration of Th17 enhances the growth of soybean plants and increases yield of potato. Thus the use of inputs such as Th17, which works at very low concentrations, will be economically wise for producers and good practice for the environment. Given that LCO containing products are already successful in the market place the cheaper production costs of Th17 (faster to produce more) and the lower concentrations required (several orders of magnitude) should make Th17 very economic. These studies, conducted on soybean and potato, show that Th17 has potential to be used as crop growth enhancer.

5.5 Acceptance or rejection of hypothesis

Hypotheses 1: For soybean, stimulation of germination caused by treatment with Th17 or LCO is greater under severely stressful conditions (low temperature) than moderately stressful conditions

Germination experiments were conducted on soybean at two different stress levels: 22 °C as moderately stressful and 15 °C as severely stressful growing temperatures. The results presented in the germinations assays in Chapter 3 showed clearly that the responses on Th17 and LCO are greater under severely stressful growing temperature conditions.

Thus, we accept hypothesis 1.

Hypothesis 2: Growth of soybean and potato crop responds more strongly to Th17 than LCO

Our results showed that the both the compounds enhance the growth of soybean and potato under various conditions of greenhouse and field. Sometimes the Th17 treatments are higher (during germination at 15 °C Chapter 3, and potato field trials Chapter 4) but sometime the effects of LCO are higher.

So we reject this hypothesis.

Chapter 6 CONTRIBUTIONS TO KNOWLEDGE

The major contributions from my work are as follows:

1. My study provides the first clear demonstration of Th17 enhancement effects on potato yield.

2. The work conducted on soybean under low temperature conditions provides a way for crop producers to help their crops deal with this stress.
    - Environmental stresses will become more frequent as climate change conditions continue to develop. Early season low temperature stress will continue to be a problem because as seasons get longer and springs start earlier crop producers will plant earlier.

3. The work also provides a clear comparison of Th17 with the already commercialized LCO technology. The comparison shows clearly that there is potential for Th17 to be developed as a commercial crop growth enhancer. There are several characteristics of Th17 which make it a better input than LCO: the bacterium producing Th17 is easier and faster to produce, and Th17 works at lower concentrations than LCO.

Chapter 7 SUGGESTIONS FOR FUTURE RESEARCH

The work that I conducted in during the two years of my thesis work has indicated additional research requirements, to facilitate the commercial deployment of Th17.

1. Investigate the mechanism(s) by which Th17 causes growth stimulation in terms of:
    - Plant hormone responses
    - Changes in plant physiology
    - Changes in gene expression
    - Changes in protein profile
2. Look for new, and more effective, compounds that are produced by other rhizobacteria and enhance plant growth at very low concentrations, to develop additional low input technologies for sustainable agriculture.
3. Explore improved commercial formulations to increase the efficacy of Th17 under field conditions.

APPENDIX

Figure 7 (a): Daily temperature reading (maximum and minimum) for the month of May and June, 2010 (growing season for soybean). The temperature is presented in °C (Source: www.climate.weatheroffice.gc.ca)

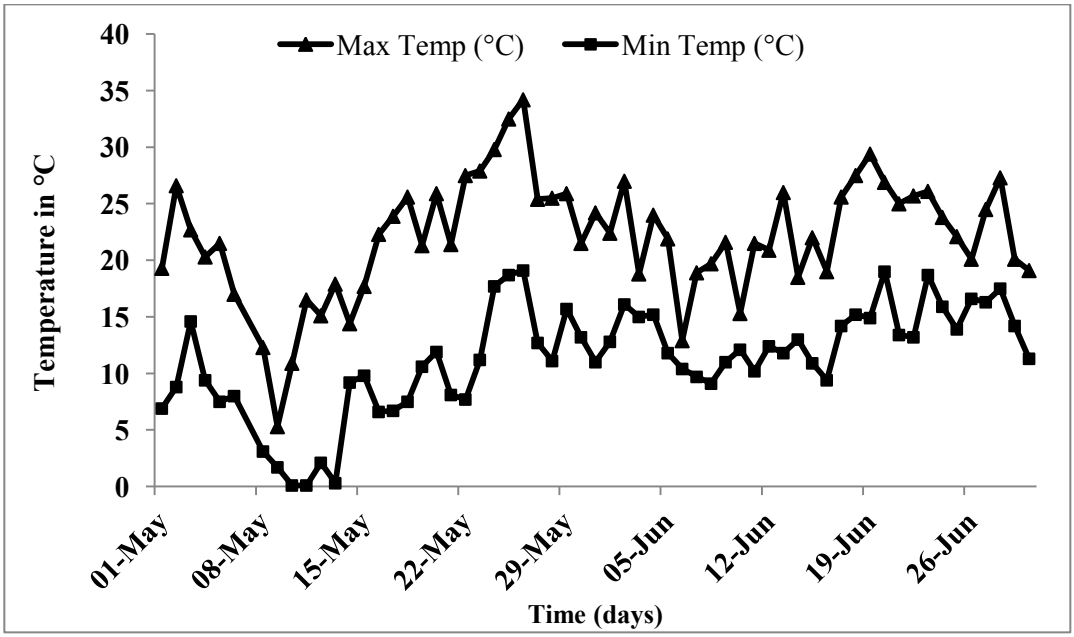


Figure 7 (b): Daily total precipitation for the month of May and June, 2010 (growing season for soybean). Total precipitation is presented in millimetres (Source: www.climate.weatheroffice.gc.ca)

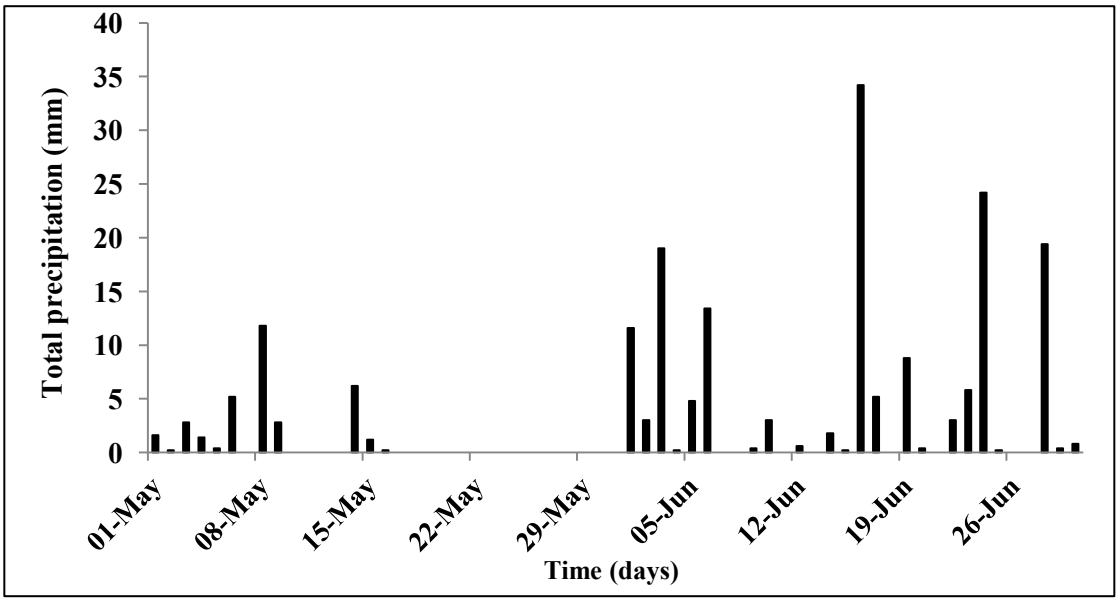


Figure 8 (a): Daily temperature reading (maximum and minimum) for the month of June and July, 2011 (growing season for soybean and potato). The temperature is presented in °C. (Source: www.climate.weatheroffice.gc.ca)

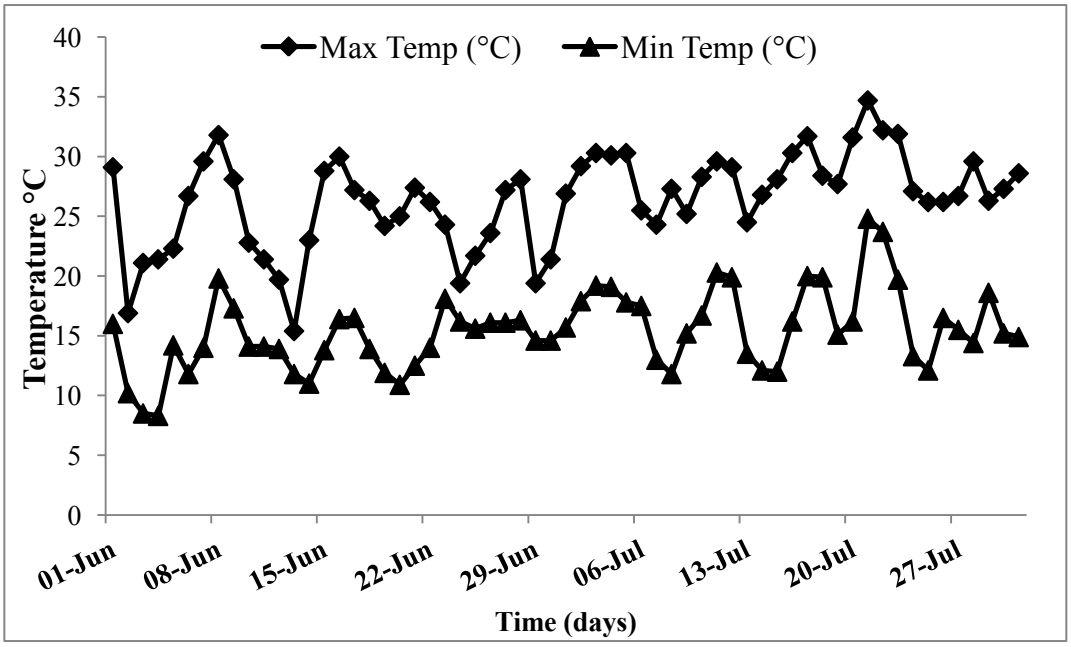
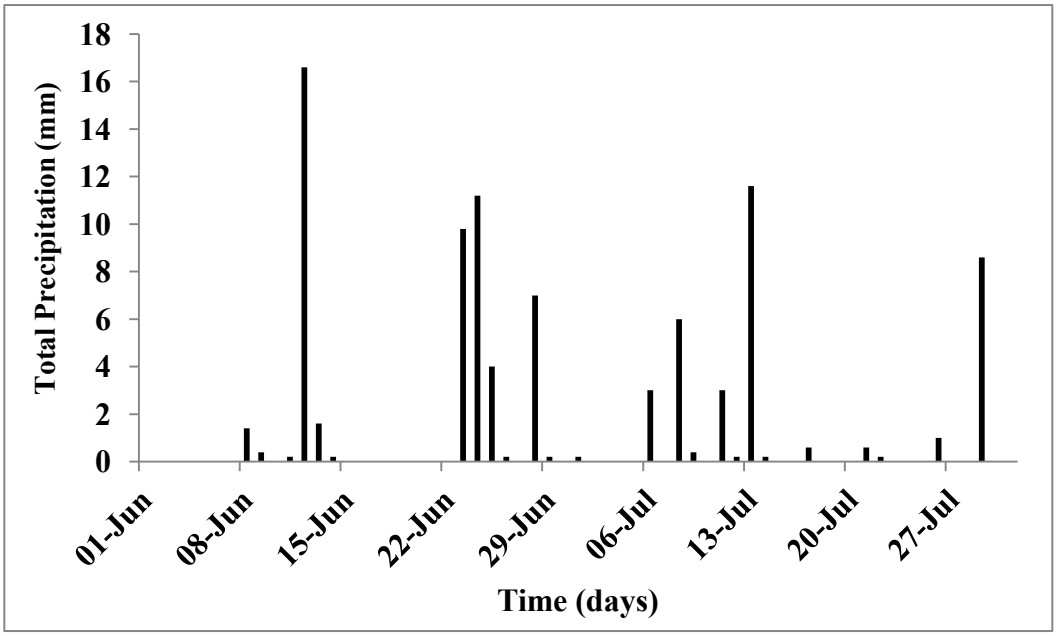


Figure 8 (b): Daily total precipitation for the month of June and July, 2011 (growing season for soybean and potato). The amount of total precipitation is presented in millimeters. (Source: www.climate.weatheroffice.gc.ca)



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