

The response of canola (*Brassica napus* [L.]) to a novel set of plant  
growth regulators: microbial signal molecules

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### III English Abstract

As a member of the Brassicaceae family, canola (*Brassica napus* [L.]) forms neither arbuscular mycorrhizal (AM) symbiotic relationships nor symbioses with rhizobia, but brassicaceous plants may detect lipo-chitooligosaccharides (LCOs), ubiquitous signal compounds that can mediate the legume-rhizobia symbiosis, and other chitin-based signals through lysin motif (LysM) receptor-like kinases (RLKs). LCOs and compounds produced by other rhizosphere microflora have been shown to promote plant growth. New agricultural genotypes of spring annual type canola cultivars 02C3, 02C6, 04C111, 04C204, Polo, and Topas, that were developed for biodiesel production, having a range of seed oil contents, were assessed for their response to LCO signal molecules produced by *Bradyrhizobium japonicum* 532C (Nod Bj V [C18:1, MeFuc]) and thuricin 17, which was produced by *Bacillus thuringiensis* non-*Bradyrhizobium* endophytic bacterium 17 (NEB17). The objective of this work was to assess the potential of these signal compounds to act as plant growth regulators of *B. napus*. The effects of treatment concentrations on germination variables were evaluated under cool, standard, and moderately high temperature conditions. The effects on emergence and surface coverage due to LCO and thuricin 17 irrigation treatments were determined using young plants grown in growth cabinet experiments. Rapid cycling *B. napus* plants are small compared to the commercial types; they can grow to their full height and produce seed after 6 weeks of growth in the controlled environments in growth cabinets (Williams and Hill 1986). The effects of acute treatment with a  $10^{-6}$  M LCO solution at planting, and subsequent foliar spray treatment, on factors contributing to yield were assessed using rapid cycling *B. napus* plants in a growth cabinet experiment. The effect of the LCO treatment on branching architecture and yield were investigated, using agricultural *B. napus* cultivars Topas and 04C111, grown under simulated cool spring and optimal temperature conditions, respectively, in greenhouse experiments. The response of *B. napus* to these signals was found to depend on the context, that is, the concentration, the temperature, the cultivar, and the salinity of the growth media; in many cases there were useful enhancements, although in some instances there were small or even negative effects, indicating the potential utility of these compounds but also that there is still much to learn. The application of  $10^{-6}$  M LCO solution to the seed at planting can accelerate emergence. Acute treatments of LCO stimulated 04C111 and Topas plants to develop ramose forms, but triggered the elongation of branches for 04C111 and reduced branch lengths for Topas. A  $10^{-6}$  M LCO solution applied to the seed at planting, and a

foliar spray at the flowering stage of plant development, stimulated higher seed weight from rapid cycling *B. napus* and Topas, respectively, as compared to untreated controls. This work has shown that  $10^{-6}$  M LCO can be applied to *B. napus* to increase factors that contribute to yield: plant emergence, branch number, and the number of siliquae produced on the apical raceme.

#### **IV Résumé en Français**

En tant que membre de la famille des Brassicacées, le canola (*Brassica napus* [L.]) ne forme ni des relations symbiotiques mycorhiziennes à arbuscules (MA) ni des symbioses avec des rhizobiums, mais les plantes de la famille des Brassicacées peuvent détecter les lipochitooligosaccharides (LCO) et d'autres signaux à base de chitine à l'aide de kinases de type réceptrices (RLK) avec le motif lysine (LysM). De nouveaux génotypes agricoles du type hâtif et annuel de *B. napus* comme les cultivars 02C3, 02C6, 04C111, 04C204, Polo, et Topas, offrant une gamme de profils d'acides gras dans les graines, ont été développés pour la production de biodiesel. Ils ont été évalués pour leur réponse à des signaux moléculaires produits par *Bradyrhizobium japonicum* 532C (Nod Bj V [C18: 1, MeFuc] ; LCO) et à la thuricine 17 produite par *Bacillus thuringiensis*, une bactérie endophyte n'appartenant pas au genre *Bradyrhizobium* de type 17 (NEB17). L'objectif de ce travail était d'évaluer le potentiel de ces signaux chimiques en tant que régulateurs de la croissance de *B. napus* dans les systèmes de production agricole. L'effet des différentes concentrations sur les variables liées à la germination des semences a été évalué dans des conditions de température fraîche, standard et moyennement élevée. Les effets de l'irrigation chronique avec du LCO et de la thuricine 17 sur l'émergence et la couverture de la surface du sol ont été déterminés à l'aide d'expériences avec de jeunes plants cultivés dans des chambres de croissance. Les effets du traitement aigu avec une solution micromolaire de LCO appliquée à la plantation et du traitement ultérieur par pulvérisation foliaire sur les facteurs contribuant au rendement ont été évalués à l'aide des plants de *B. napus* à cycle de croissance rapide dans une expérience utilisant les chambres de croissance. Les effets du traitement avec le LCO sur les ramifications architecturales des plants et le rendement ont été étudiés en utilisant un cultivar agricole et un cultivar expérimental qui ont été cultivés dans à une température optimale et une température simulant un printemps frais dans la serre. Il a été observé que la réponse de *B. napus* à ces signaux dépend du contexte : ce sont la concentration, la température, le cultivar et la salinité des milieux de croissance qui jouent le rôle principal. Dans de nombreux cas, il y avait des améliorations utiles, mais dans certains cas, il y avait aussi des effets négligeables, voire négatifs. Cela indique que ces composés ont une utilité probable, mais aussi que plusieurs interrogations persistent. L'application de la solution micromolaire de LCO sur les graines avant l'ensemencement peut accélérer l'émergence et produire des jeunes plants multifoliés et pétiolés ayant le potentiel de dominer les autres plants dans un champ. Les

traitements aigus avec du LCO des plants de 04C111 et Topas ont stimulé le développement des formes avec des branches multiples. La solution micromolaire de LCO appliquée sur les graines à planter a produit un accroissement du poids des graines et un rendement plus élevé des plants de *B. napus* ayant un cycle de croissance rapide et du cultivar Topas par rapport aux témoins. Ce travail a démontré que le  $10^{-6}$  M LCO peut être appliqué au *B. napsus* pour accroître les facteurs qui contribuent au rendement : l'émergence de la plante, le nombre de branches et le nombre de siliques produits sur le racème apical.

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## VI Preface & Contribution of Co-Authors

The contents of sections 3 to 6, inclusively, are drawn from manuscripts for publication, co-authored by myself, Alfred Souleimanov, Pierre Dutilleul, and Donald L. Smith.

Donald Smith, my supervisor at Macdonald College, acquired funding from the Green Crop Network and the Eastern Canadian Oilseed Alliance, and provided laboratory and greenhouse space, oversaw the experiments, provided guidance, reviewed the manuscripts, and acquired the students who helped to collect siliquae and seed data.

Alfred Souleimanov provided guidance, isolated the LCO and thuricin 17, and provided the text describing the production method (Sections 3.3.2 and 3.3.3).

Pierre Dutilleul edited Sections 3.3.5, 4.3.5, 5.3.2, and 6.3.3, and was consulted with respect to many details of the statistical methodology and the description of the statistical methods.

## VII Contributions to Knowledge

This thesis describes an investigation of the interactions between *B. napus* genotypes and three microbial signal molecules, the potential new plant growth regulators: lipo-chitooligosaccharide, thuricin 17, and chitopentaose.

1. The optimum concentration of LCO, for the stimulation of 75 % more germinated *B. napus* cv. Polo seeds during the 5 to 15 growing degree day period, is  $10^{-6}$  M (Fig. 3.1), under AOSA (1993) standard temperature conditions: 20 °C 16 h day/ 30 °C 8 h night for *B. napus*. This is important because it indicates that LCO is effective in dilute solution. The lipid moiety confers a propensity for LCO to form spherical arrays (micelles) in aqueous solution (Groves et al. 2005). The hydrophobic region of the micelle is enclosed by a polar shell. As such, micelles of LCO may have different signaling properties to the monomeric form. Pure LCO is unlikely to aggregate at micromolar concentrations (Groves et al. 2005). The micromolar level of concentration was previously identified by Goedhart et al. (2000), who worked with common vetch (*Vicia sativa* L.), as the concentration at which the binding sites in root hair cell walls become saturated with LCO. At micromolar concentrations, lipo-chitooligosaccharides induced the expression of the isoflavone reductase gene, a marker gene of the isoflavonoid synthesis pathway in

microcallus cell suspension cultures derived from alfalfa (*Medicago sativa* subsp. *sativa*, genotype RA3) (Savouré et al. 1994). Zhang and Smith (2001) reported that spraying field *B. napus* plants with micromolar concentrations of LCO leaves caused a transient increase in photosynthetic rate.

2. There is a temperature by treatment interaction involving chitopentaose. Germination was enhanced by about 17.2 %, *i.e.*, about 1 more germinated seed per count, for each Petri dish of 25 *B. napus* seeds that were irrigated with a standing solution of  $10^{-6}$  M chitopentaose under 24 h 30 °C conditions, but not at lower temperatures. This understanding of an effect of a microbial associated molecular pattern (MAMP) on *B. napus* growth is an incremental advance for agroecological science. It is important for agricultural practice, specifically the seeding rate, because 50 % of planted *B. napus* seeds do not emerge under field conditions (Harker 2003). Fast and early germination, emergence, and crop canopy closure can provide weed-inhibiting light conditions at the soil surface to reduce herbicide inputs, reduce selection pressure for weed resistance, improve yield, improve quality, and increase agricultural *B. napus* profitability (Silvertown 1980).
3. Eleven days after planting (DAP), the presence of a root from the stem node or hypocotyl, *i.e.*, an adventitious root, was significantly and positively correlated to treatment with  $10^{-6}$  M LCO solution, for cultivar 04C111 (Table 4.2). Previously, Voesek et al. (1999) reported the development of adventitious roots from *B. napus* cv. Chikuzen shoot tissue, and Topas to a lesser extent, grown largely in stagnant/unaerated agar solution. Niknam and Thurling (1995) found significant variation between *B. napus* genotypes in the number of adventitious roots formed in response to waterlogging. This is important because, at a critical growth stage, adventitious roots could conceivably confer an advantage to plants exposed to nutrient, water, or biotic stress (Dodd et al. 2010; Ghanem et al. 2011).
4. Nanomolar ( $10^{-9}$  M) thuricin 17, included in  $200 \times 10^{-3}$  M NaCl agar-based growing

media in plant culture vessels, increased the tolerance of 04C111 plants, as indicated by the 58.9 mg increase to fresh weight, 38 DAP at 24 h 10 °C. This supports recent reports of thuricin 17 affecting plant growth under abiotic stress conditions (Subramanian 2013), and should be followed up by further research (see Section 10).

5. The application of 10 mL  $10^{-6}$  M LCO solution to the seed at planting enhanced rapid cycling *B. napus* emergence, after 1 week of growth at 24 h 25 °C, by 28.1 %. The seed treatment increased the proportion of rapid cycling plants that produced seeds by 15.4 % (Fig. 5.1), and increased the seed weight produced per plant by 2.009 mg, which was a 60.8 % increase. It seems that this seed treatment overcomes many of the negative temperature effects previously observed. For example, similar temperature conditions have been previously reported to inhibit megagametophyte development and flower production, and render some *B. napus* cultivars entirely sterile (Morrison 1993; Omid et al. 2010).
6. There were significant genotypic differences in the effects of LCO treatment on factors that contribute to yield. LCO-seed-treated 04C111 plants grew approximately 1 more branch, on average, under optimal temperature conditions (Fig. 6.1) in a greenhouse, as compared to plants grown from untreated seed. The total branch length of LCO-sprayed 04C111 plants was 16.93 cm longer ( $\pm 8.41$  standard error,  $p_{\text{diff}} = 0.0425$ ), and therefore apical dominance was decreased by 17.94 % (Figs. 6.3a and b). However, the longest primary branches formed by Topas control plants were 13.43 cm longer ( $\pm 3.14$  standard error,  $p_{\text{diff}} = 0.0394$ ) than the longest branches formed by LCO-sprayed Topas plants (Fig. 6.6b). Topas plants that were sprayed with LCO produced 9.1 ( $\pm 2.5$  standard error,  $p_{\text{diff}} = 0.0193$ ) more siliquae, and 76.1 % higher ratios of apical:lateral siliquae (Fig. 6.6c and d). Although *B. napus* is frequently discussed in the scientific literature as if it was a uniform and homogeneous entity (e.g., Farina et al. 2012), there is substantial evidence for its heterogeneity particularly due to its allopolyploidy, and the fact that *B. napus* is known to have several centres of origin. For example, gene homeologs, such as those for Nodulation Signaling Pathway1 NSP1 and NSP2, derived from genomes A and C in *B. napus*, have divergent expression profiles with preferential activity in the roots of the A-

genome copy (Hayward et al. 2012). Liang et al. (2013) and Subramanian (2013) described the detection and response by *Arabidopsis* to Nod factor treatments. There are other factors that would seem to contribute to the uniformity of *B. napus* in its response to microbial signal molecules. For example, the low glucosinolate trait derived originally from a single genotype, the Polish spring oilseed rape variety Bronowski (Kondra and Stefansson 1970), and the trait of self-compatibility has been attributed to Westar, which contributed its susceptibility to blackleg (*Leptosphaeria maculans* [Desmaz.] Ces. and De Not) to many of the cultivars currently used in agriculture.

7. Unsprayed 04C111 plants grown from untreated seed in a high temperature greenhouse produced approximately 7 more seeds, which was a 51.44 % increase compared to water-sprayed plants that were grown from seed to which  $10 \text{ mL } 10^{-11} \text{ M}$  thuricin 17 was applied at planting (Fig. 6.4). Therefore, at the concentration used for the seed application, thuricin 17 has the potential to decrease *B. napus* yield. This is an exciting finding because thuricin 17 induced plant defense-related enzymes such as ascorbate peroxidase in soybean (*Glycine max* [L.] Merr. cv. OAC Bayfield), and *Arabidopsis* plants lacking ascorbate peroxidase 2 produced more seeds under prolonged heat stress (Jung et al. 2011; Suzuki et al. 2013). Therefore, a hypothesis remains to be confirmed (see Section 10): that thuricin 17 induces ascorbate peroxidase in *B. napus*, thereby inhibiting hydrogen peroxide signaling that would otherwise induce the protection of reproductive tissue under heat stress conditions.

## **1 Introduction**

The rationale of the research included in this thesis was to evaluate a new class of potential plant growth regulators for application to canola (an increased demand for canola was effectively mandated by The Renewable Fuels Regulations (Government of Canada 2013)) and investigate the potential of an agriculture system for Canada to reduce greenhouse gas emissions, by using bacteria-sourced plant growth regulators to enhance the growth, development, and yield of *B. napus* cultivars for biodiesel and edible oil production. The broader context of this research is discussed in section 1.1.1 below.

### **1.1.1 The context of agricultural economy and biofuels**

Biofuels are a contentious technology and the subject of a vigorous socially and environmentally significant public debate in the popular media and in the Canadian Parliament. Much of the discussion arises from: the scientific quantification of net energy expenditures; nitrous oxide emission associated with untimely application of nitrogen fertilizer in conventional agriculture; and the appropriation of land for large-scale monocultures, which would otherwise be forested or used for the production of food, as opposed to commodity production. The public debate regarding biofuels – at this time – vilifies the agrofuel (*e.g.* the Canadian Biotechnology Action Network 2013), where an agrofuel is defined as a fuel based on a crop otherwise grown in large-scale industrial monocultures to produce agrifood, such as conventional Canadian Prairie wheat production. Society and economies need biofuels to bridge the energy gap between fossil fuel scarcity and the nascent sustainable energy society. Biofuels allow for such a transition because they can function within the current fossil fuel-oriented infrastructure. Of the available liquid fuel options, biodiesel offers the benefits of a superior energy balance and flexibility in application for both transportation and home heating. By offering diversification in production responsive to strong demand markets, the producers growing *Brassica* for localized systems of biodiesel processing, such as was proposed by Pacala and Socolow (2004), can participate in the long-term transition from fossil fuel-era infrastructure and thereby bring stability to rural communities.

The definition for biofuel is proposed here to deviate from agrofuel, in that biofuel is produced ethically on small polyculture fields, or fields with multiple canopies of intercropped species, in

long-term, complex rotations, which include the sequential planting of annuals such as potato (*Solanum tuberosum* [L.]), grains such as maize (*Zea mays* [L.]), Brassicas, and perennials such as willow, poplar, or nitrogen-fixing alder. Among other field rotation designs, Zegada-Lizarazu and Monti (2011) suggested that a *B. napus* crop could be followed by flax (*Linum usitatissimum* [L.]) and then safflower (*Carthamus tinctorius* [L.]) or sunflower (*Helianthus annuus* [L.]).

Global contexts of increasingly intense climatic events, energy scarcity, political instability, inadequate policy, and the vagaries of prices in the global market, have combined to make agricultural production risky to a prohibitive degree. Over-reliance on a very narrow portfolio of energy supply stocks has already resulted in increased consumer costs and fluctuations in available supply. This has prompted serious interest (*e.g.*, Government of Canada 2013) in broadening the energy supply and manufacturing demands to include agrofuels from agricultural feedstocks. Alternatively, to meet this demand, biofuels may be grown and processed domestically and in “cradle-to-cradle” cycles similar to the internal processes of a living organism, which enable materials to maintain their status as resources. Cradle-to-cradle industrial cycles accumulate intelligence, *i.e.*, the quickness and superiority of their degree of apprehension, over time (McDonough 2002; Braungart et al. 2006). However, the biodiesel market in Canada is subject currently to the prohibitively high price of domestically-sourced canola, due to the reliance of the industry on increasingly expensive fossil fuel production systems that require fossil fuels at every stage, that is, for farm machinery, fertilizer, *etc.*

Biofuels hold much promise in decreasing net greenhouse gas emissions, because the CO<sub>2</sub> emitted from their combustion has been recently fixed from the atmosphere via plant photosynthesis. In contrast, combustion of fossil fuels releases CO<sub>2</sub> acquired from the atmosphere hundreds of millions of years ago, resulting in greater net increase in present-day atmospheric greenhouse gasses. Nonetheless, lifecycle analyses of the plant feedstocks that are used to produce agrofuels often indicate negative or only marginally positive responses in terms of greenhouse gas emissions (Janulis 2004; Ulgiati 2001). This is largely due to the fact that agrofuel feedstock production is heavily dependent upon nitrogen fertilizer to maximize yield and heavy farm machinery to cultivate and harvest it (all run on fossil fuels). However, biodiesel derived from oilseed crops, particularly *Brassica* spp., is one of the most efficient alternative fuels (Baquero et al. 2011).

### **1.1.2 The experimental cultivars**

The *B. napus* cultivars used in these trials experiments were: Polo, Topas, 02C3, 02C6, 04C111, and 04C204. The spring-type cultivar, Polo, was bred by Danisco for high oil concentration (Rahman et al 2001). Topas is a Swedish (SWAB, Svalöv, Sweden) spring cultivar with relatively low seed oil content, compared to the others. 02C3, 02C6, 04C111, and 04C204 were bred for high oleic acid seed oil contents, using conventional crosses and pedigree, for the Green Crop Network (Peter McVetty, University of Manitoba). Beaudette et al. (2010) reported that, in conventional agricultural systems, the microbial biomass was higher where 04C204 and Topas were grown, compared to sites where Polo was grown (Beaudette et al. 2010). In Beaudette et al.'s experiments, 04C204 sites produced higher nitrous oxide emissions due to greater rates of release of organic compounds, by 04C204 plant roots to their surrounding environment. Such rhizodeposition can alleviate carbon limitation among heterotrophic denitrifying bacteria (Beauchamp et al. 1989). The experimental cultivars are introduced in more detail in Section 2.2.

The experiment described in Section 5 employed rapid cycling *B. napus* plants. Rapid cycling *Brassicas* were developed as efficient and inexpensive tools for education and research (Williams and Hill 1986). They are capable of producing ten or more generations per year (Aslam et al. 1990). To provide results, rapid cycling *B. napus* plants required neither a field season, nor space in a greenhouse.

### **1.1.3 *Brassica napus* under stress**

Severe heat stress can lead to hyperfluidization and disruption of membranes, inactivation of proteins by unfolding, misfolding, and aggregation, and the accumulation of reactive oxygen species (e.g. Volkov et al. 2006; Sharma et al. 2010). During the heat stress response, heat stress proteins (Hsps), many of which are chaperones, accumulate (Finka et al 2011). Chaperones can prevent protein aggregation or restore misfolded and aggregated proteins to correctly folded polypeptides, which can recover afterwards their active conformation for normal function (Sharma et al. 2010). Furthermore, the inefficient conversion of sucrose to phloem-unloaded, degraded sucrose hexoses (glucose and fructose) is a key limiting step for ovary development. Inadequate sucrose import, and its degradation within reproductive organs, cause fruit and seed abortion under heat stress conditions (Liu et al 2013). Trehalose-6-phosphate, sucrose non-

fermenting kinase-1, and the target of rapamycin kinase complex, modulate nutrient signaling and metabolic processes where sugar availability is low (Liu et al. 2013), and the growth rate of pollen tubes through the style is limited by an inadequate supply of sucrose, glucose, and fructose in the pistil, at least for cotton (*Gossypium hirsutum* [L.] cv. ST4554B2RF) (Snider et al. 2011). As a consequence, many researchers (e.g., Young et al. 2008) have observed *B. napus* seed set to be dramatically reduced under heat stress conditions. A more in depth discussion of heat stress will be found in Section 2.3.

In cold environments, plants display a self-defence mechanism called acclimation. Cold acclimated plants show changed physiology, biochemistry, molecular states, and altered energy requirements. Cold acclimation has been accounted for by other groups by measured increases in cell size in the mesophyll leaf tissue, which is found between the upper and lower epidermal layers, or increases in the number of (palisade) layers of pillar-shaped photosynthetic cells below the leaf upper epidermis (Gorsuch et al. 2010; Dahal et al. 2012). Cold acclimation during germination and cellular dehydration are correlated with high levels of ABA, which results in increased levels of hydrophobic and thermostable dehydrins, which provide cellular protection during osmotic stress, too (Rapacz et al. 2013). The response of *B. napus* to cold is discussed further in Section 2.4, below.

Broadly speaking, plant growth responds to salinity in 2 phases: a rapid osmotic phase that inhibits the growth of young leaves, and a slower ionic phase that accelerates senescence of mature leaves (reviewed by Rahdari and Hoseini 2011). Water flows passively because of differences in water potential, which is the ability of water to do work. Water flows from areas of high water potential to areas with low water potential. Therefore, a leaf must have a lower or negative potential relative to the root. Decreased plant water potential results in decreased water use efficiency (Rahdari and Hoseini 2011). The *B. napus* leaf water potential has been observed to decrease in response to  $200 \times 10^{-3}$  M NaCl (Akbari et al. 2011). The experiments described below include saline levels of growth media, up to  $400 \times 10^{-3}$  M NaCl, which is just below the usual salinity level of seawater ( $\sim 460 \times 10^{-3}$  M NaCl). Furthermore, the xylem vascular tissue transports water and ions, including sodium ions, to the leaf blade, where these ions inhibit enzyme activity and disturb metabolism. For example, Jafarinia and Shariati (2012) reported that the acceptor site of electron transport in PSII was influenced more by salinity, as compared to the

donor site of electron transport. Ion accumulation also coincides with the up-regulation of voltage-dependent anion-selective channel protein, which forms a channel to allow diffusion of small hydrophobic molecules (Cheng et al. 2012). The effects of salinity on *B. napus* are discussed in more detail in Section 2.5.

#### **1.1.4 Microbial plant growth regulators**

Lipo-chitooligosaccharide, thuricin 17, and chitopentaose are proposed in this thesis as potential microbial growth regulators for *B. napus*. Previously, Noel et al. (1996) attributed longer roots of *B. napus* cv. Westar seedlings (90 h after seeding) to microbial plant growth regulators indole-3-acetic acid and cytokinin, produced by wild-type *Rhizobium leguminosarum*. Bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves ethylene precursor ACC and thereby lowers the ethylene contents of developing or stressed plants, was shown to lengthen primary roots produced by *B. napus* cv. Westar plants treated with strain UW4 (identified by various groups as either *Enterobacter cloacae* or *Pseudomonas putida*) after 7 days of growth (Cheng et al. 2009). Similar results were found for 5 day-old *B. campestris* plants primed with ACC deaminase-containing *Methylobacterium fujisawaense* suspensions (Madhaiyan et al. 2006). Pallai et al. (2012) reported the lengthening of *B. napus* cv. 45A71 roots by *Pseudomonas fluorescens* 6-8 and G20-18, which produce cytokinins and ACC deaminase. Phosphate solubilizing bacterium *Bacillus megaterium* A6 increased the mean of *B. napus* cv. Zhongyou 821 shoot dry weights on a per pot basis, and % total nitrogen per plant, 60 DAP (based on Fig. 2 from Hu et al. 2013). Other natural and synthetic growth regulators of *B. napus* are introduced in Sections 2.6 and 2.7.

#### **1.1.5 Experimental design and regression modeling**

The statistical methods used to describe seed germination include Gompertz's (1825) regression model, which is based on a nonlinear function, which is described in Section 3.3.5. The Gompertz function was chosen because it has been used to describe monotone growth in a number of environmental and biological applications (Winsor 1932; Piegorsch and Bailer 2005). The text below also refers to the Central Rotatable Composite Design (CRCD), which is explained in the second paragraph of Section 4.3.2. The CRCD was chosen because it is an

efficient method to identify optimal points of interaction, and treatment levels are determined mathematically. The number of tests required for CRCD includes the standard  $2^k$  ( $k$  = number of variables) factorial with its origin at the center, replicate tests at the center, plus  $2k$  points fixed axially at a distance from the center to generate quadratic terms (Aslan 2008). The axial points are chosen such that they allow rotatability, which ensures that the variance of the model prediction is constant at all points equidistant from the design center (Box & Hunter 1957). Replicates of the test at the center are very important as they provide an independent estimate of the experimental error. The optimal points of interaction, which are reported as results in Section 4.4.2, were stationary points that were maxima, *i.e.*, each were the highest point at a peak (Figs. 4.4, 4.5, and 4.6). The ridges of maximum response were calculated along the radius for each variable. These values were used to predict optimal combinations. Other methods for statistical analysis are described in Sections 3.3.5, 4.3.5, 5.3.2, and 6.3.3.

## 1.2 Hypotheses

The overall hypothesis of this work was that lipo-chitooligosaccharide (LCO), thuricin 17, and chitopentaose can produce positive changes in plant growth that lead to increased yield of *Brassica napus* plants. That is to say, extremely low concentration solutions of LCO, thuricin 17, and chitopentaose, applied as either one-time seed treatments, or as one-time foliar sprays, or as standing solution, or in agar-based growth media, produce changes in the growth, development, architecture, and the yield of experimental *Brassica napus*. More specific hypotheses were:

**Section 3:** Novel plant growth regulators lipo-chitooligosaccharide (LCO), thuricin 17, and chitopentaose can enhance the germination of canola (*Brassica napus* [L.]) cultivars. Multiple germination assays using *B. napus* cultivars developed for biodiesel production were conducted under controlled environment conditions in growth cabinets. The effects of 3 signal molecules on germination parameters are reported.

1. LCO, thuricin 17, and chitopentaose are highly conserved molecular signatures characteristic of their respective classes of microbes, but absent from *B. napus*, and are as such Microbe-Associated Molecular Patterns (MAMPs, reviewed by Boller and Felix 2009) that are hypothesized to stimulate the germination of *B. napus* seed as estimated based on Gompertz function parameters (Gompertz 1832).

2. The experimental, high seed oil content *B. napus* genotypes (02C3, 02C6, 04C111, 04C204, Polo) are more likely to survive and germinate more rapidly and uniformly, compared to a low oil control genotype (Topas); and lipo-chitooligosaccharide, thuricin 17, and chitopentaose will increase survival and the speed and uniformity of germination under stressful cold (10 °C) and warm (30 °C) temperature conditions, as estimated based on Gompertz function parameters.

**Section 4:** Irrigation with lipo-chitooligosaccharide Nod Bj V (C18:1, MeFuc) and thuricin 17 solutions regulates the the phyllotaxy, height, and mass of young canola (*Brassica napus* [L.]) plants. In this section, short term factorial, Randomized Complete Block, and Central Rotatable Composite Design experiments investigated the effects of LCO and thuricin 17 on plants grown in controlled environment chambers. Hypotheses 1 to 4 refer to *B. napus* cvs. 04C111 and Topas, grown in peat pellets and irrigated by standing solution. Hypotheses 5 and 6 refer to 04C111 plants grown in agar-based media in plant culture vessels.

1. Irrigation treatment with LCO and thuricin 17 will increase the number of 04C111 plants emerged from seeds, under 10/4, 25/20, 30/30 °C conditions.
2. Irrigation treatments, with LCO or thuricin 17 solution, are hypothesized to increase plant growth, quantified by plant height.
3. The morphogenic effect on plant growth, as a result of treatment with LCO or thuricin 17, is hypothesized to increase under stressfully low (10/4 °C) and high (30/30 °C) temperature conditions to produce multifoliate and petiolate forms, quantified by counting the number leaves and measuring the length of the petioles.
4. Optimal points for the interaction of LCO concentration with temperature are hypothesized to exist and be identifiable by ridges of maximum response on a response surface. The response of Topas to irrigation with LCO solution will be quantified by architectural metrics such as stem height, primary root length, petiole lengths, and leaf number.
5. The presence of thuricin 17 in the growth media is hypothesized to increase the tolerance of 04C111 plants to saline conditions. Tolerance will be quantified by plant fresh and dry weight, height, and leaf number.

6. The presence of thuricin 17 in the growth media will increase plant growth, quantified by the rate of embryogenesis and the extension of petiole lengths.

**Section 5:** A micromolar concentration of lipo-chitooligosaccharide (Nod Bj V [C18:1, MeFuc]) regulates the emergence and seed productivity of rapid cycling canola (*Brassica napus* [L.]) plants. Randomized Complete Block experiments investigated the effects of the application of LCO to the seed, and the effects of a supplementary treatment, which included both the application of LCO to the seed and an LCO spray at the flowering stage of plant development, on rapid cycling *B. napus* plants that were grown under moderately high temperature conditions (24 h 25 °C) in controlled environment chambers.

10. Application of 10 mL  $10^{-6}$  M LCO to rapid cycling *B. napus* seed at planting is hypothesized to accelerate the emergence of plants. Lipo-chitooligosaccharide seed treatment is hypothesized to increase the production of rapid cycling *B. napus* seed, quantified by seed number and seed weight after harvest.
11. Application of a supplementary  $10^{-6}$  M LCO foliar spray, in addition to the application of 10 mL  $10^{-6}$  M LCO to the rapid cycling *B. napus* seed at planting, is hypothesized to increase the production of seed, and increase yield at harvest, after growth under stressful (24 h 25 °C) temperature conditions.

**Section 6:** The response of canola (*Brassica napus* [L.]) agricultural cultivars to *Bradyrhizobium japonicum* 532C signal molecule lipo-chitooligosaccharide (Nod Bj V [C18:1, MeFuc]) and *Bacillus thuringiensis* endophytic bacterium 17 bacteriocin. Three greenhouse experiments investigated effects owing to the application of LCO, to the seed and to the leaves, of 04C111 and Topas, grown under optimal and simulated “cool Spring” conditions, and the effect of the application of thuricin 17 to 04C111 seed in a high temperature greenhouse.

12. An application of  $10^{-6}$  M LCO to seed at planting, supplemented with a  $10^{-6}$  M LCO foliar spray at the flowering stage of plant development, will increase 04C111 branch number, branch length, and factors that contribute to 04C111 yield (siliquae, seeds per siliqua, and seed weight) after growth to harvest in an optimal temperature greenhouse.

13. It is hypothesized that the application of 10 mL  $10^{-11}$  M thuricin 17 solution to 04C111 seed at planting, supplemented with a foliar spray at the flowering stage of plant development, will increase factors that contribute to yield (siliquae, seeds per siliqua, and seed weight) in a high temperature greenhouse system.
14. Under simulated “cool Spring” conditions in a greenhouse, it is hypothesized that the application of  $10^{-6}$  M LCO to seed at planting, supplemented with a  $10^{-6}$  M LCO foliar spray at the flowering stage of plant development, will increase factors that contribute to Topas yield (branch number, branch length, number of siliquae, seeds per siliqua, and seed weight) and seed quality (number of apical siliquae, apical ratio, oil % d.w.).

### **1.3 Objectives**

When elaborating the experiments, several objectives were defined:

#### Section 3

1. The first objective is to determine the parameters of seed germination, which will be estimated from Gompertz functions for the cultivars of interest, which will be supplied with LCO, thuricin 17, and chitopentaose in solution.
2. The second objective is to determine whether or not the interaction effects of cultivar-by-temperature and signal-by-temperature have significant effects on the Gompertz function parameters, and indices derived from them.

#### Section 4

3. To determine, in a peat pellet growing system, whether or not the emergence of 04C111 plants will be increased, after 11 days of growth, due to irrigation with LCO or thuricin 17 solution, at 10/4, 25/20, and 30/30 °C.
4. To determine 04C111 height, leaf number, and petiole lengths, after irrigation with LCO or thuricin 17 solutions, at 10/4, 25/20, and 30/30 °C.
5. To determine, using Topas grown in a peat pellet system, the optimal points for the interaction of LCO concentration with temperature by finding ridges of maximum response in a Central Rotatable Composite Design experiment.

6. Quantify embryogenesis, final fresh and dry weights, plant height, petiole extension, and leaf number of 04C111 plants treated with thuricin 17 and grown in plant culture vessels, in saline media.

## Section 5

7. To assess whether or not the application of 10 mL  $10^{-6}$  M LCO to rapid cycling *B. napus* seed, and the application of a supplemental  $10^{-6}$  M LCO foliar spray, will increase factors that contribute to yield under moderately high (24 h 25 °C) temperature conditions.

## Section 6

8. To grow 04C111 under optimal temperature conditions in a greenhouse, and to assess the effect of the application of 10 mL  $10^{-6}$  M LCO solution to the seed, and to assess the effect of  $10^{-6}$  M LCO foliar spray on floral initiation, architecture, and factors that contribute to yield.
9. To assess the effect of the application of 10 mL  $10^{-11}$  M thuricin 17 solution to the seed on factors that contribute to yield, in a greenhouse system where 04C111 is grown under high temperature conditions
10. To assess the effect of the application of 10 mL  $10^{-6}$  M LCO to Topas seed, and subsequent spray treatment with  $10^{-6}$  M LCO solution, on floral initiation, architecture, and factors that contribute to yield, and seed quality, in a greenhouse system where Topas is grown under simulated cool spring conditions.

As a result, the thesis consists of 4 papers: the first focuses on the beginning of development, *i.e.*, germination; the second focuses on subsequent growth and early development; the third focuses on development right through to yield, but using a rapid system; and the fourth focus on development, including plant architecture through to yield with conventional genotypes.

## 2 Literature Review

### 2.1 *Brassica napus*

Cultivation of Brassicaceae as oil crops can be traced back several thousand years in Asia and North Africa (Dixon 2007). The earliest literary references to sarson (*Brassica rapa* L., formerly known as *B. campestris*, whereas gobhi sarson denotes *Brassica napus* L.), date from approximately 1500 BC (Singh 1958). Other evidence of the use of this plant in antiquity was unearthed by Colombini et al. (2005), who found oil from Brassicaceae seed in lamps dating from the 500 to 700 BC, from the Roman age of Egypt. Such seed oils of Brassicaceae are slow-burning and odourless. The seed oil of Brassicaceae fueled European institutional alcove lamps, which were ubiquitous for the duration of the Middle Ages (Dixon 2007).

The *B. napus* genome is a hybrid summation of the diploid *B. rapa* and *Brassica oleracea* (L.) genomes (U 1935). As such, *Brassica napus* is an allotetraploid with 19 haploid chromosomes and a 1130 to 1240 megabasepair genome derived from *B. oleracea* ( $n = 9$ , approximately 500 mbp) and *B. rapa* ( $n = 10$ , approximately 600 mbp) (Barret et al. 1998; King 2007). Some varieties, e.g., *sahariensis*, have origins in the Mediterranean region, where *Brassica oleracea* was originally confined (Tsunoda 1980; Burkill 1985). However, as a hybrid, *Brassica napus* has several centres of evolution. There is molecular evidence that interspecific hybridizations of *B. oleracea* and *B. rapa* occurred several times naturally and domestically (Palmer et al. 1983; Allender and King 2010). Wild forms of *B. napus* grow in Denmark, The Netherlands, New Zealand, Britain, and on the beaches of Gothland in Sweden (Rakow 2004; Dixon 2007).

Oilseed *B. napus* includes annual and biennial types, which are referred to alternatively as spring and winter types. The biennial common rape is referred to as *B. napus* L. ssp. *oleifera* Metzg. *Brassica napus* comprises at least two other forms that have been recognized as subspecies: *B. napus* ssp. *rapifera* Metzger (= subsp. *napobrassica* [L.] Hanelt.) also known as rutabaga or swede has fleshy roots and rarely escapes cultivation; and *B. napus* ssp. *napus*, also known as oilseed and vegetable rape, has slender roots and is commonly naturalized and occurs as a weed (King 2007). The focus of this thesis is the spring type of the oilseed form.

*Brassica napus* may be distinguished visually from other Brassicaceae based on the partial clasping of the stem by the auricles of the lower leaves, and the elevation of the floral buds

above the open flowers on the terminal raceme (Bengtsson et al. 1972). The plant and its stages of growth are described more precisely in the sections below. It contains 1 to 2 % more seed oil than *B. rapa* and has the highest seed yield potential of the Brassicaceae crops, under favourable environmental conditions (Downey and Klassen 1979; Kimber and McGregor 1995).

Although science has known *B. napus* since the nineteenth century, systematic research on *B. napus* only began in the late 1930s (Juska and Busch 1994). At that time, many countries developed national policies to promote self-sufficiency in production of fats and oils. Commercial cultivation of rapeseed for production of lubricants for marine engines was supported by Canadian federal government research since 1942 (Wanasundara 2011). In the early 1970s, responding to the implication of erucic acid in cardiac necrosis and inhibition of fatty acid oxidation in the hearts of rats (Christopherson and Bremer 1972; McCutcheon et al. 1976), Canadian breeders produced genetic material with <2 % erucic acid in the seed oil, in terms of 1996 standards, and containing <30 µmol of glucosinolates of any one or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate and 2-hydroxy-4-pentenyl glucosinolate per gram of air-dried, oil-free solid meal at a moisture content of 8.5 % (Canola Council of Canada 2013). The roots of mature plants, and the leaves and roots of seedlings still contain large amounts of biologically active glucosinolates (Stefansson and Hougen 1964). In 1979, the Canadian low erucic acid varieties were the first from which “Canadian oil low acid” canola was developed. Canola has since been developed from other species. *Brassica napus* is often referred to as Argentine canola, and *B. rapa* is referred to as Polish canola.

The cultivated area of *B. napus* is expanding rapidly in Canada, in part owing to the intensifying demand for biodiesel feedstock (Franzaring et al. 2008). In 2000, the average agricultural *B. napus* yield in Canada was 1.561 Mg ha<sup>-1</sup> (Statistics Canada 2012). From 2008 to 2010, this value was 1.892 Mg ha<sup>-1</sup> (Harker et al. 2012b). Approximately 300,000 bee (*Apis mellifera* [L.]) colonies contribute to its pollination (Munawar et al. 2009). Appropriate field rotations, which include *B. napus* for sustainable food and fuel production in Europe, were reviewed by Zegada-Lizarazu and Monti (2011).

### **2.1.1 Stages of *Brassica napus* growth**

The life cycle of *B. napus* can be divided into developmental stages: germination and emergence;

leaf production; stem extension; flower bud development; flowering; silique development; seed development; leaf senescence; stem senescence; and silique senescence (Sylvester-Bradley 1985). These stages are considered to overlap, for example, the vegetative, generative, and reproductive organ stages overlap (Diepenbrock 2000).

#### **2.1.1.1 Germination and emergence**

*Brassica napus* seeds are dark brown to black, light brown, or reddish and 1.8 to 3 mm in diameter (Warwick 2013). *Brassica napus* produces orthodox nondormant dry seed, which is to say that it is able to germinate after a drying period or at low temperatures. Typically, for 6 to 9 h after planting, the seeds swell physically without developing. The imbibition phase is accompanied by the establishment of basic metabolic activity (Schopfer and Plachy 1984). The axis of the embryo straightens before true radicle growth commences. The elastic expansion of the whole embryo is reversible at this stage (Schopfer and Plachy 1985). Seeds at this stage of development can still be dried back without losing their germination vigour. The seed coat is finely reticulate-alveolate, thin and fragile, and when wetted it lacks the typical cruciferous mucilage. The seed coat absorbs insignificant amounts of water during germination and does not hinder radicle protrusion (Schopfer and Plachy 1984).

The splitting of the testa by the expanding cotyledons of imbibed *B. napus* seed is the first morphological indication of germination (Schopfer and Plachy 1984). Radicle protrusion depends on growth driven by water uptake. The emergence of the radicle is considered to be the completion of germination. Following this stage, desiccation tolerance is lost irreversibly (Schopfer and Plachy 1984). The germination phase is characterized by a second increase in both water uptake and respiration, indicative of the embryo's expansion and growth. The mean time required for *B. napus* germination has been shown to be correlated with field performance and seed yield (Larsen et al. 1998). After germination, water uptake increases and storage proteins and lipids are utilized to support seedling growth (Nykiforuk and Johnson-Flannagan 1997).

Challenges to *B. napus* germination include: autotoxicity, light, lack of moisture, salinity, seed characteristics, soil compaction, and temperature conditions. *Brassica napus* produces autotoxic allelochemicals, e.g., the powdered seed and root of *B. napus* var. Kirariboshi contain both volatile and water-soluble compounds capable of inhibiting the germination of Kirariboshi seed

(Yasumoto et al. 2011). Continuous white light is also inhibitory, particularly at low temperatures and low water volume, but increasing the amount of water can almost totally eliminate light-induced inhibition (Bażańska and Lewak 1986). A lack of sufficient moisture in the vicinity of the seed can induce high water stress and inhibit *B. napus* germination on the soil surface (López-Granados and Lutman 1998). Sodium chloride concentrations are also known to progressively inhibit *B. napus* seed germination (Shahbazi et al. 2011). Not all salts inhibit germination equally, and cultivars respond differently to salinity (Farhoudi et al. 2012). Seed oil components can also challenge *B. napus* germination, for example, transgenically modified high-stearate *B. napus* has disadvantageous germination characteristics, relative to untransformed controls (Linder et al. 1998). Furthermore, *Brassica napus* cvs. that produce large seeds normally have better adaptability to harsh conditions during germination (Geritz et al. 1999; Adamski et al. 2009). Compacted soils impede radical emergence and thereby inhibit *B. napus* germination (Shaykewich 1973). Once the seed has imbibed water, soil temperature is the main factor affecting its transition to the radicle emergence stage (Nykiforuk and Johnson-Flannagan 1997). The baseline temperature for seedling growth is approximately 5 °C, and germination rarely occurs below 2 °C (Nykiforuk and Johnson-Flannagan 1997). For rapid germination, dry *B. napus* seed requires only imbibition at a suitable temperature, e.g., 25 °C (Kucera et al. 2005). Kondra et al. (1983), in laboratory studies, showed that 90 % of *B. napus* cvs. seed germinated at 2 to 25 °C. The germination time varied with temperature, from 11 to 14 days at 2 °C to 1 day at 21 to 25 °C. Although Timiriaziff (cited by Coffman 1923) reported the germination of brassicaceous seed in a block of ice, the optimum temperatures for *B. napus* are in the range of 10 to 30 °C, and low temperature conditions slow the rate of imbibition, damage *B. napus* embryos, and prevent germination (Omidi et al. 2010). Other work has shown that the germination response of different cultivars to low temperature conditions has marked nonlinearities (Marshall and Squire 1996). The seed of some *B. napus* cultivars remain viable but do not germinate under low temperature conditions. There have been links made between poor performance under cool temperature conditions and low glucosinolate character, but the enzymatic hydrolysis of glucosinolates results in a number of products with potential to inhibit *B. napus* seed germination (Acharya et al. 1983; Brown and Morra 1996).

Emergence is a function of soil temperature, seed germination, and early seedling growth, and

the high germination rate (90 %) of high quality *B. napus* seed greatly overestimates actual seedling emergence (often ~50 %) under field conditions (Habekotté 1997; Harker et al. 2003). There is a strong positive association of precipitation with *B. napus* emergence density (Harker et al. 2012a). Whereas seed is usually targeted for planting at 2 cm deep, actual planting depths are variable owing to irregular surfaces, the variable depths of crop residue on no till or low till fields, and mechanical variability. There is, however, a tendency for greater *B. napus* emergence where it is seeded 4 cm below a very dry surface soil layer (Harker et al. 2012a; Gao et al. 1999). Under other extreme or average conditions, *Brassica napus* emergence density is greater from a depth of 1 versus 4 cm. (Harker et al. 2012a; Gao et al. 1999). A high seeding rate and good stand establishment reduce weed populations and have positive effects on yield (Burnett et al. 2003). It should be noted that, in Eastern Europe, the sprouts of *B. napus* at this early stage of plant development are used as a seasoning (Burkill 1985).

#### **2.1.1.2 Leaf production**

Once above the ground, *Brassica napus* cotyledons expand and produce chlorophyll for photosynthesis. The shoot apex initiates typically 10 to 15 leaves, lacking stipules, in an alternate or helical arrangement. There is minimal internode elongation during vegetative growth, and a rosette body plan forms before stem elongation. Morrison and McVetty (1991) reported a leaf initiation rate (plastochron) of 46 growing degree days (GDD) above 5 °C, or about 66 GDD above zero, after their plants had produced approximately 7 leaves, at a rate of about 4 d per leaf appearance (phyllochron). The blade of a basal leaf is lyrate-pinnatifid and more or less pinnately lobed (Warwick 2013). The leaf surface is glaucous and glabrous or sparsely hairy when immature. The waxy leaf surface may restrict ion leakage (Waalén et al. 2011). Depending on genotype and a minimum leaf number the shoot apex becomes responsive to photoperiod and vernalization cues and switches identity from a vegetative meristem to a floral meristem. Before flowering, the interception of radiation is the main determinant of developmental rate, but after flowering, temperature is the main determinant (Hodgson 1978). The middle and distal cauline leaves are sessile, the blade bases are auriculate or amplexicaul, with entire margins (Warwick 2013). Large plants with a greater leaf surface are capable of greater photosynthetic activity and more photosynthetic products may be directed to seed formation (Dewey and Lu 1959). Many groups have found that well watered plants produce the highest leaf area indices (*e.g.*, Tesfamariam et al. 2010).

The microsomal cytochrome P450 monooxygenase KLUH/CYP78A5 protein, first known as a maternal regulator of seed size, also promotes the growth of leaves and floral organs and prolongs the plastochron (Anastasiou et al. 2007; Wang et al. 2008). Based on its non-cell autonomous mode of action, which does not seem to involve any of the known phytohormones, the protein was suggested to be involved in the generation of a unique mobile growth stimulator (Adamski et al. 2009). Other natural and synthetic plant growth regulators are discussed in a separate section below.

#### **2.1.1.3 Stem extension**

Under optimum growing conditions, *Brassica napus* can exceed 120 cm in height (Kirkland 1992). The synthetic plant growth regulators most widely applied to agricultural *B. napus* block gibberellin (GA) and inhibit excessive growth of the stem that can result in lodging. Lodging can otherwise reduce yield and make the harvesting of *B. napus* difficult, compared to *B. rapa*. Stem elongation involves the lengthening of the internodes between each leaf to 5 to 10 mm. *Brassica napus* plants normally expand 15 to 20 internodes at this stage (Potter 2009). Quantitative trait loci controlling shoot architecture have been identified in a number of species, including other, smaller brassicaceous plants, (*Arabidopsis thaliana* [L.] Heynh.) (Pérez-Pérez et al. 2002).

*Brassica napus* growth is very plastic and the plants readily fill light niches in open canopies (McGregor 1987; Angadi et al. 2003). Lateral branches arise from secondary shoot apical meristems laid down in the axils of leaves. Axillary meristems can either remain dormant or activate to produce a shoot branch (Shimizu-Sato and Mori 2001). Branching patterns are determined normally by genotype, light, and nutrients. The influence of genotype and the environment on *Brassica* development and shoot architecture have ultimate area-specific implications for cv. selection and yield.

Stand density and applied nitrogen are well known factors that influence the total numbers of branches and the numbers of secondary branches (*e.g.*, Jixian and Hua 1997). The number of branches per plant has also been closely correlated with soil moisture during the growing season (Saini and Sidhu 1997; Halvorson et al. 2001). Physiologically, branching is regulated by hormones including cytokinin and auxin and abscisic acid (ABA) (Cline 1991; Chatfield et al.

2000). Cytokinin promotes axillary bud outgrowth. It can stimulate cell division activity when applied directly. Auxin, transported from the apical bud, inhibits the expression of a subset of genes that encode the isopentenyltransferase enzyme, which catalyzes the first committed step to cytokinin biosynthesis. In Arabidopsis, the more axillary branching gene, *MAX4*, is involved in the production of a carotenoid derivative signaling molecule that suppresses branching of the shoot (Sorefan et al. 2003).

#### **2.1.1.4 Flower bud development and flowering**

The Brassicaceae typically have simple and indeterminate inflorescences. From the main inflorescence at the apical meristem, floral initiation propagates basipetally to the lateral inflorescences (Tittone 1988 and 1990 in Jullien et al. 2011 [en français]). The flowers open in acropetal sequence on each inflorescence, and remain open and vital for ~3 days (Nedić et al. 2013). Flowering on the late-developing secondary inflorescences may continue for some time after the main stem has finished flowering (Wang et al. 2011). This means that the basal and oldest ramifications bear the youngest inflorescences. Compared to the lower branches of *B. napus*, the upper branches produce fewer flowers (Habekotté 1993). The flowers of *B. napus* generally have a typical cruciferous structure, four free sepals and petals that widen at the apical part, representing a suitable landing platform that enables pollinators to collect pollen and nectar easily (Nedić et al. 2013). The petals are monochromatic, golden, or creamy to pale yellow, broadly obovate with rounded apices and no visible UV guide marks for insect pollinators other than the fluorescent anthers (Nedić et al. 2013). Four of the stamens produce anthers above the corolla, the other two stamens are hidden inside the cup-like petal bases (Nedić et al. 2013). The flowers have a gynaecium with two fused carpels and a superior ovary (Warwick et al. 2009). Many hundreds of flowers can be produced by an individual *B. napus* plant. However, many can fail to reach maturity, owing to the inhibition of pollination by cold and damp conditions. The numbers of flowers on *Brassica* inflorescences has been increased by external applications of benzyladenine (Morgan et al. 1983). It should be noted that, for some growers, the plant material of interest is the edible flowering section, which is known in northeastern Portugal as *grelos* (Batista et al. 2011). Furthermore, the yellow flowers function as morphological signals to attract common pollen beetles (*Meligethes aeneus* F. and *Meligethes viridescens* F.) (Médiène et al. 2011).

The flowers are mainly visited by bees (Hymenoptera: Apidae) and hoverflies (Diptera: Syrphidae) (Bommarco et al. 2012). Field experiments have established that the presence of pollinating insects, especially *A. mellifera*, impact *B. napus* productivity and yield beneficially (Koltowski 2002; Rosa et al. 2011). Pollination can occur when an insect or other object touches the anthers and small clouds of pollen burst out (Eisikowitch 1981). The individual ovules in each silique must each be fertilized by a pollen grain. The stigma is mature when the flower opens, encouraging cross-pollination initially, but the anthers mature later, encouraging self-pollination (Delaplane and Mayer 2000). *Brassica napus* is generally 60 to 70 % self-pollinated (Williams 1978).

*Brassica napus* plants that grow vigorously develop sufficient leaf area at flowering, maximize light interception, have low flower densities, limit light reflectance and absorption by petals during seed development, and hence produce higher seed yields (Harker et al. 2012a). Therefore, breeders have selected for small petals and apetalous flowers to improve the absorption of light by the green canopy (Habbekotté 1997a). Other groups have found that the duration of flowering can determine *B. napus* seed yield (Campbell and Kondra 1978; Johnston et al. 2002). Plants with larger vegetative structures that flower late, but produce more siliques, can produce higher yields (Mendham and Salisbury 1995). However, delayed flowering can increase the risk that the late summer heat will reduce reproductive efficiency and yield (Young et al. 2004; Kutcher et al. 2010). Double haploid lines, such as those used to produce the open pollinated cultivars that were used in the work reported here, typically flower early and mature late. These attributes can contribute to higher yields, particularly in a single crop monoculture. However, in Canada, delayed maturity increases the probability that early frost will result in more green seed and reduced seed oil quality and yield. In general, flowering is the most critical stage influencing the yield of rapeseed and it is the period that the crop is most susceptible to stress (Richards and Thurling 1978). The effects of temperature stress on *B. napus* flowers will be discussed further below.

#### **2.1.1.5 Silique and seed development: components of yield**

The silique is the pod (or envelope or hull) with a seed or seeds in it. Siliques are pods with seed, whereas pods do not necessarily contain seed. Within each inflorescence, siliques develop acropetally (Wang et al. 2011). Older siliques at the base of a *B. napus* inflorescence are well

developed while new flowers are initiating at the tip. Wang et al. (2011) reported that the early developed siliquae had a competitive advantage over later formed siliquae, and that the early siliquae could produce larger numbers of seeds per silique. Jullien et al. (2011) divided the development of siliquae into two phases: the heterotrophic phase, when each pod is supplied mainly by leaf and stem photosynthesis; and the autotrophic phase, when each pod is supplied by its own photosynthesis. Jullien et al. (2011) subdivided the autotrophic phase into phases, *i.e.*, one when photosynthesis is efficient and at its maximum, during which time biomass is allocated primarily to the pods; and the second phase, when photosynthetic efficiency declines and biomass is allocated primarily to the seeds.

Seed yield and seed oil content as quantitative traits are the result of genotype, environment, and genotype-by-environment interaction (Enqvist and Becker 1993; Gunasekera et al. 2006). The seed yield per plant of *B. napus* has three components: the number of siliquae, the number of seeds per silique, and individual seed weight (Leul and Zhou 1998; Fan et al. 2010). Yield is positively correlated with low cellular respiration in some plant species, and selection for energy use efficiency, which is under epigenetic control in *B. napus* plants, resulted in increased yield (Hauben et al. 2009). Seed yield and oil content are measurements of the adaptation of a cultivar to an environment (Moghaddam and Pourdard 2011), but a number of genes that control seed size in *Arabidopsis* have been identified and characterized (*e.g.*, Ohto et al. 2009; Adamski et al. 2009).

*Brassica napus* seeds are relatively small, 0.1 to 0.2 mm in diameter and 250 000 seeds kg<sup>-1</sup>. Seed size is an important aspect for the storage and accumulation of the products of photosynthesis. As such, seed size determines seed storage capacity by limiting the complement of oil and protein bodies (He and Wu 2009).

Seed growth and development are affected by the position of the silique on the plant (Diepenbrock and Geisler 1979). Compared to the lower branches of *B. napus*, the upper branches produce fewer siliquae with fewer but larger seeds (Habbekotté 1993). Late-forming siliquae form smaller seeds (Clarke 1979). Larger seeds produce more vigorous seedlings with larger cotyledons (Major 1977). Seed size may also be influenced by the resource status of the

plant and those with a higher biomass are likely to have larger seeds (Venable 1992; Gambin and Borrás 2009). Seed size and number of seeds per silique can also be affected by plant density, irrigation, and seeding date (Clarke 1979). In any case, the variance of weight of mature dry seed from *B. napus* is large and a Coefficient of Variation of 50 to 60 % is normal (Austin 1972; Araneda-Durán et al. 2010).

Norton and Harris (1975) divided the development of *B. napus* seed into three phases. During the first phase, in the early stages of seed development, lipids with a fatty acid composition resembling that of photosynthetic tissues account for a small proportion of the dry matter. The onset of storage lipid biosynthesis during *B. napus* seed development coincides with a qualitative and quantitative change in fatty acid composition (Kater et al. 1991). During this phase of storage lipid biosynthesis, the enzyme activities of the components of the fatty acid synthase system increase rapidly. The second phase of seed development, identified by Norton and Harris (1975), was the onset of embryo development. During embryo development, monounsaturated eicosenoic (C<sub>20:1</sub>) and erucic (C<sub>22:1</sub>) acids appears, and oleic (C<sub>18:1</sub>) acid increases transitorily. Storage oil and proteins account for ~40 and ~20 % of the dry matter, respectively, by the end of the second phase. During the third phase of seed development, seed weight more than doubles, and sugars are transferred from the hull to the seed to support a continued rise in erucic acid content, but the dry matter composition and the proportions of other fatty acids remain constant (Norton and Harris 1975). The transition of the zygotic embryo to the dry seed stage is accompanied by a reduction in ABA and many free gibberellic acids (GAs), especially the bioactive 3 $\beta$ -hydroxylated GAs (Hays et al. 2007). Levels of 3-deoxy GAs remain relatively high.

#### **2.1.1.6 Senescence**

Maturation begins with a transition phase, during which the switch from maternal control to filial control occurs (Weber et al. 2005). Deng and Scarth (1998) found that 30 °C days and 25 °C nights hastened seed maturation by 10 to 15 days, compared to 15 °C days and 10 °C nights. When storage compounds have accumulated, water content has decreased, and desiccation tolerance is established, seed maturation is completed. Norton and Harris (1975) identified an inverse relationship between the disappearance of aqueous soluble constituents from the pod, and the accumulation of oil and protein. The seed's oil and protein contents are negatively

correlated, usually, although yellow-seeded varieties have both higher oil and protein contents (Hu et al. 2013). The effects of temperature on seed oil content and quality are discussed further below.

### **2.1.2 The seed oil of *Brassica napus***

Seed oil content and quality are variable. They depend on climatic conditions, fertilization, tillage practices, the place of the seed within the silique, the location of the silique on the plant, the time of the season when they mature, and so on (*e.g.*, Omid et al. 2010; Gao et al. 2010). Seed oil content may be affected negatively by increasing plant density (Momoh and Zhou 2001). Fatty acid compositions of seed oil vary between high and low silique positions (Bechyne and Kondra 1970; Diepenbrock and Geisler 1979). Plant diseases, such as *Alternaria*, reduce both seed size and oil content (Prasad et al. 2003). And, despite the fact that *B. napus* seeds typically contain high amounts of oil in temperate or cold climates, higher temperatures during seed development can result in elevated levels of total saturated fat (Scarth and McVetty 1999). Similarly, microspore-derived embryos matured at 35 °C showed elevated cotyledonary saturated fatty acid levels, increased monounsaturated oleic acid contents, and reduced quantities of linoleic and linolenic acids when compared to those matured at room temperature (Beaith et al. 2005).

In Fawcett et al.'s (1994) study, greenhouse-grown *B. napus* plants contained only about 40 % as much fatty acid as field grown plants. Wild type *B. napus* oil consists of about 50 % erucic acid (22:1), 8 % eicosenoic acid (20:1), 15 % oleic acid (18:1), 17 % linoleic acid (18:2) and 10 % linolenic acid (18:3) (Yuan et al. 2008). Most cultivars normally produce oil with 55 to 65 % oleic (18:0), 14 to 20 % linoleic, and 8 to 12 % linolenic acid (Hu et al. 2006; Javidfar et al. 2006). The different uses of *B. napus* have given rise to specialized cultivars, high erucic acid rapeseed (HEAR) and low erucic acid rapeseed (LEAR), for example. The rest of this section provides a summary of the incompletely understood processes by which *B. napus* biosynthesizes its seed oil.

Glycolytic intermediates, the products of metabolized sucrose from the phloem, such as hexose-phosphates, phosphoenolpyruvate, and pyruvate, are imported into the plastid and used for fatty acid synthesis (Vigeolas et al. 2007). Seed plastids metabolize pyruvate and glucose-6-

phosphate (G6P) preferentially to synthesize oil, unlike leaf chloroplasts which use acetate (Slabas et al. 2001). Glucose-6-phosphate is converted to pyruvate through the nonoxidative steps of the pentose phosphate pathway, and 3-phosphoglycerate (PGA) is formed by Rubisco. Pyruvate can be transformed into acetyl-CoA and CO<sub>2</sub> in the plastid or the mitochondrion, via the pyruvate dehydrogenase enzyme. Carbon dioxide is reassimilated into PGA by Rubisco (Schwender et al. 2004). This reassimilation provides 20 % more acetyl-CoA for fatty acid synthesis and 40 % less loss of carbon as CO<sub>2</sub> than would otherwise be the case. Fatty acid biosynthesis is catalysed by two enzymes: acetyl-CoA carboxylase, which produces malonyl CoA; and a type II, dissociable fatty acid synthetase.

Within the plastid, fatty acids are synthesized *de novo* from acetyl-CoA in a series of condensation and elongation reactions. A pool of mostly 16:0- and 18:0-acyl chains linked to acyl carrier protein (ACP) is formed by the actions of ketoacyl ACP synthetases I, II, and III (Barker et al. 2007). Seed material contains 30 times more 18:0-ACP desaturase mRNA than young leaves.

Stearoyl-ACP can be exported to the cytoplasm for incorporation into complex lipids or desaturated by a soluble ferredoxin-dependent  $\Delta 9$ -desaturase, to produce 18:1-ACP. Stearoyl-ACP  $\Delta 9$ -desaturase ensures that only 18:1-ACP of the unsaturated fatty acids is in high abundance. This initial desaturation reaction occurs exclusively in the plastid. Manipulation of the activity of  $\Delta 9$ -desaturase has resulted in a decrease in the level of unsaturated lipids in *B. napus* seed oils (Slabas et al. 2001). Acyl-ACP thioesterases A and B cleave acyl-ACP to free fatty acid and ACP. Short-chain acyl-ACP thioesterase has been used to produce up to 60 % lauric acid in *B. napus* seed oil (Burgal et al. 2008). Oleoyl-ACP proceeds to membrane lipid synthesis in the plastid by the so-called prokaryotic pathway, or is acted upon by thioesterase to produce free oleic acid, which is exported from the plastid. *B. napus*'s thioesterase selectively metabolizes 18:1-ACP, although it can also use 16:0 ACP, or any number of fatty acid ACPs.

In order to be available for passage into the cytoplasm, free oleic acid, CoASH, and AcylCoA synthetase produce 18:1-CoA at or near the outer plastid envelope. The other fatty acids pass into the cytoplasm similarly. Desaturation, elongation, and phospholipid/acyl-CoA exchange

mechanisms further modify the acyl chains (Barker et al. 2007). This happens in the cytosol, primarily in the endoplasmic reticulum. The extra carbon commonly found in Brassicaceae elongated acyl chains originates from a distinct cytosolic acetyl-CoA pool, probably derived from mitochondrial citrate metabolism. The acyl-CoA pool can be modified to produce complex lipids. Unsaturated acyl-CoAs can be elongated by membrane bound fatty acid synthetase to produce erucic acid. The synthesis of erucic acid in *B. napus* is controlled by the fatty acid elongation 1 (*FAE1*) locus. The trait is controlled by two highly homologous genes: *Bn-FAE1.1* (*E1* locus) that codes for  $\beta$ -keto-acyl-CoA synthase and *Bn-FAE1.2* (*E2* locus) (Barret et al. 1998; Cullen et al. 2008).

At high pathway flux, incorporation of 18:1 into TAGs by acyltransferases is favored, whereas the influence of the desaturase enzymes is increased under conditions of reduced oil biosynthesis (O'Hara et al. 2007). Oleic acid content is controlled by a major locus called *fatty acid desaturase 2* (*fad2*). *Fad2* encodes the endoplasmic  $\Delta$ -12 oleate desaturase enzyme that desaturates oleic to linoleic acid by insertion of a *cis*-double bond in the  $\Delta$ -12 ( $\Omega$ -6) position (Scheffler et al. 1997; Schierholt et al. 2001). There are four loci of the *fad2* gene on four different linkage groups, two of the *B. rapa* genome and two on the *B. oleracea* genome.  $\Delta$ 12-desaturase is frequently the target of transgenic experimentation (e.g., Tso et al. 2002). A single nucleotide mutation, substituting a leucine for a proline, in the *fad2* gene on the *B. rapa* genome, linkage group N5, can cause an increase in the oleic acid content (Schierholt et al. 2001).

Two major loci controlling linolenic acid content were found to correspond to two membrane-associated fatty acid desaturase, *fad3*, genes. One is located on each of the *B. napus* parent genomes (Barret et al. 1999). *Fad3* encodes for endoplasmic  $\Delta$ 15-desaturase, which is responsible for the desaturation of linoleic into linolenic acid. ABA has a high activity for the induction of  $\Delta$ 15-desaturase genes (Qi et al. 1998).

Glycerol-3-phosphate (G3P) is synthesized from the glycolytic intermediate dihydroxyacetone phosphate via G3P dehydrogenase, or from glycerol via glycerol kinase (Vigeolas et al. 2007). The supply to developing seeds of G3P co-limits oil accumulation. The Kennedy Pathway begins with the sequential acylation of G3P, using saturated or unsaturated acyl-CoA, by G3P-

acyltransferase (GPAT) to lysophosphatidic acid, then by lyso-phosphatidic acyltransferase (LPAT) to produce phosphatidic acid (Furukawa-Stoffer et al. 2003).

LPAT excludes 22:1-CoA and other unsaturated acyl from the second position on the glycerol backbone, which is referred to by its stereospecific numbering position, *sn*-2. The resultant phosphatidic acid is dephosphorylated by phosphatidic acid phosphatase (PAP) to release diacylglycerol (DAG). Burgal et al. (2008) added a transgene encoding a short-chain LPAT and thereby increased seed oil laurate content by 5 %. Transgenic expression of a mutated yeast *sn*-2 acyltransferase (*SLC1-1*) in industrial rapeseed cv. Hero resulted in increased seed oil content and very long chain fatty acids present in the seed oil under field conditions (Taylor et al. 2001).

Endoplasmic reticulum localized diacylglycerol acyltransferase (DGAT) or, in the absence of acyl-CoA, phospholipid:DAG acyltransferase acylates DAG to produce TAG, and a reversible transacylase can transfer an acyl group from one DAG to another (Murphy 2004). DGAT may limit TAG formation when lipid accumulation rates are high (Perry et al. 1999). Over-expression of *DGAT1* results in substantial increases in oil content and seed size (Sharma et al. 2008). TAG is synthesized in the endoplasmic reticulum and stored in oil bodies formed through budding (Katavic et al. 2006).

An ideal oil composition is low saturated fatty acids (<6 %), high oleic acid (>50 %), moderate linoleic (<40 %) and low linolenic (<14 %) (Iqbal et al. 2011). The profile of conventional *B. napus* oil is dominated by oleic acid rich TAGs (Mottram et al. 1997). As such, oleic acid levels typically correlate directly with oil content in *B. napus* (O'Hara et al. 2007). Plants subjected to 30 °C day, 25 °C night for 40 days produced seed high in oleic acid (Deng and Scarth 1998). Such a treatment is considered a moderately high temperature treatment. The relative proportion of oleic acid in seed oil increases until full maturity. Seed oil with increased levels of oleic acid, in combination with reduced linoleic and linolenic acids, shows high oxidative stability without extensive hydrogenation and relatively low levels of oxidation products (Scarth and McVetty 1999). Since high oleate oil shows such high oxidative stability, when compared to other vegetable oils, it is thermostable; less susceptible to oxidative changes during refining, storage, and frying; and it can be heated to higher temperatures without smoking (Schierholt et al. 2001). There are two separate pools of oleate in *B. napus* seeds, which are used in the process of seed oil biosynthesis to form either eicosenoic or erucic acid (Kroumova and Wagner 1997). Nosheen

et al. (2011) found that *B. napus* plants treated with *Azotobacter vinelandii* produced seed oil with more oleic acid.

Linoleic acid is unsaturated, with two double bonds, which provide nutritional benefits (Barker et al. 2007). The fatty acid at the second *sn* position of the TAG molecule is generally unsaturated, and at that position, on the glycerol molecule, linoleic takes priority over oleic acid (Mottram et al. 1997). Deng and Scarth (1998) found intermediate temperatures during seed development resulted in a high linoleic acid content in the seed oil. Linoleic and linolenic acids are essential polyunsaturated fatty acids that need to be supplemented in the diet, but they are vulnerable to oxidation (Newton 1998).

Linolenic acid is noted for low oxidative stability, and thus has deleterious effects on the shelf life of *B. napus* oil. Standard *B. napus* seed oil contains 8 to 10 % linolenic acid. Higher linolenic acid content occurs under lower temperature regimes (15/10 °C) (Deng and Scarth 1998). Linolenate levels rose with longer exposure to low temperatures (Deng and Scarth 1998). However, the same study found the effect of temperature to vary among cultivars. Linolenic acid content is also influenced by photoperiod during seed development.  $\alpha$ - and  $\gamma$ -linolenic acid are  $\Omega$ -3 and  $\Omega$ -6 linolenic acid, respectively. The isomers are essential dietary fatty acids. Liu et al. (2001) introduced  $\Delta$ 6-desaturase and  $\Delta$ 12-desaturase into *B. napus* plants.  $\Delta$ 6-desaturase converts linoleic to  $\gamma$ -linolenic acid.  $\Delta$ 12-desaturase converts oleic to linoleic acid. The resultant plant seed oil contained 43 %  $\gamma$ -linolenic acid. Similarly, Tso et al. (2002) found the recombinant expression of  $\Delta$ 12- or  $\Delta$ 6-desaturases produced seed oil with 30 to 45 %  $\gamma$ -linolenic acid. Nosheen et al. (2011) found higher linolenic acid contents for *B. napus* plants treated with *Azospirillum brasilense*.

In the leaf, plant defense mechanisms are stimulated by the metamorphosis of linolenic acid to jasmonic acid (JA) via the action of enzymes in the oxylipin pathway, starting with the oxygenation of free  $\alpha$ -linolenic acid by 13-lipoxygenase (Schaller 2001; Creelman and Mulpuri 2002). As such, and owing to its role in photosynthesis and pollen development, the elimination of linolenic acid from seed oil is probably impossible (Barker et al. 2007). Microspore-derived embryos had about 35 % more linolenic acid than the zygotic embryos of mature seeds (Wiberg et al. 1991).

Erucic acid is seed-specific and is confined almost exclusively to the neutral lipid fraction in developing oilseeds (Taylor et al. 1991). Seed oils high in erucic acid are desirable as industrial feedstocks for the production of cosmetics, high fluidity lubricants, medicinal products, nylon, plasticizers, surfactants, water repellents, and waxes (Thormann et al. 1996). Although there are no indications of erucic acid toxicity in humans, it is known to cause cardiac lipidosis and necrosis in rats (Laryea et al. 1992). In Canada, only oils with <5 % erucic acid are permitted for edible use. Two gene loci control erucic acid content in *B. napus* (Dorrell and Downey 1964; Chen and Heneen 1989) *Brassica napus* plants treated with *A. vinelandii* can produce 4 % more seed oil, with less erucic acid and glucosinolate in the oil (Nosheen et al. 2011).

## 2.2 The experimental cultivars

The experiments reported in this thesis employed *B. napus* cultivars Polo, Topas, and open pollinated *B. napus* lines made using conventional crosses and pedigree for the Green Crop Network (Peter McVetty, University of Manitoba). The spring-type Polish canola, Polo, is a conventional, double low, *i.e.*, low glucosinolate and low erucic acid, cultivar. Polo and Marita produced the least pollen of the cultivars investigated by Kotowski (2001), who classified them as winter cultivars.

Topas (SWAB, Svalöv, Sweden) is a commercial-quality cultivar. Coventry et al. (1988) reported that Topas seed typically achieved 8 to 10 % germination, but increased to 95 to 100 % when subjected to cold (4 °C) for 10 days. They found older embryos germinated at a higher rate when subjected to cold (Coventry et al. 1988). El-Beltagi and Mohamed (2010) analyzed Topas seed, and they found 35.4 mg g<sup>-1</sup> dw phenolic content, most notably sinapic acid and its derivatives, and 8.83 %  $\alpha$ -linolenic acid (18:3), 0.91 % erucic acid, 20.5 % total saturated fatty acids, 78.99 % total unsaturated fatty acids, and 73.02 mg hydrophobic antioxidant tocopherol (vitamin E) per 100 g oil, which was the lowest level of tocopherol among the cultivars studied by their group, but the highest among the cultivars studied by Salvati et al. (2011). Topas was included recently in diversity and linkage disequilibrium analyses by Delourme et al. (2013). Topas was included initially in our experiments as a comparative control because of its relatively low seed oil content, but recent work has shown that Topas performs well in Nova Scotian agricultural systems (Kevin Vessey, Saint Mary's University, personal communication).

The crosses that produced high erucic acid *B. napus* sister cvs. 04C111 and 04C204 are presented

in standard breeder's notation, where '/' represents one cross, and increasing numbers of '/' marks indicates previous crosses, here: HiQ///Apollo///86LL141//Tatyoan/R83-14. The HiQ ancestor was a doubled haploid spring-type *B. napus* line (Stringam et al. 2000). The crosses that produced sister cvs. 02C3 and 02C6 are presented in standard breeder's notation, here: Polo///86LL141//Tatyoan/R83-14. The crosses that produced Topas: Bronowski//Gulle/Hermes.

### **2.3 Abiotic stresses to *B. napus* growth: high temperature stress**

The optimum daily temperature for *B. napus* flowering is 20 °C (Chen et al. 2005). *Brassica napus* is most susceptible to stress during the flowering stage of plant development (Richards and Thurling 1978). Heat stress can considerably reduce the number of flowers. Qaderi et al. (2010) found that 28 °C day / 24 °C night, as compared to 22 °C day / 18 °C night, decreased root, stem, leaf, and total plant biomass. Even merely moderately high temperature conditions, such as a daily mean temperature of 25.5 °C, can result in abnormal megagametophyte development and inhibit *B. napus* flower production (Polowick and Sawhney 1988; Omid et al. 2010). Morrison (1993) reported *B. napus* cvs. Westar and Delta grown at a daily mean 22 °C in growth cabinets were almost entirely sterile. However, Fan and Stefansson (1986) found that, for two types of cytoplasmic male sterile *B. napus*, anthers of flowers became at least partially male fertile in 26 °C days / 20 °C nights and 30 °C days / 24 °C nights. High temperatures during seed development can result in elevated oleic acid contents, and increased temperature and water stress can reduce the total seed oil concentration and quantities of linoleic and linolenic acids (Hocking and Stapper 2001; Beaith et al. 2005).

### **2.4 Abiotic stresses to *B. napus* growth: cold temperature stress**

*Brassica napus* is usually seeded in the early spring months, when the temperature is well below optimum (Nykiforuk and Johnson-Flanagan 1999). Dry *B. napus* seed requires only imbibition at a suitable temperature for rapid germination (Kucera et al. 2005). In keeping with Levitt (1980), who found that decreased hydration of tissues follows the inhibition of cell expansion, Yao et al. (2005) found the expression of dehydrin genes can be induced in germinating seeds under low temperature conditions. Yao et al. (2005) found that after 5 days of germination at 5 °C, germination rates were only 4 and 15 %, for two *B. napus* varieties. Nykiforuk and Johnson-Flanagan (1999) concluded that low (6 °C) temperature conditions slow the rate of imbibition, damage embryos, and prevent germination. They found slow and asynchronous

germination and seedling growth lead to poor stand establishment and reduced yield (Nykiforuk and Johnson-Flanagan 1999). At 10 °C, Nykiforuk and Johnson-Flanagan (1999) found the water uptake rate lagged, but the delay in germination did not damage the seed or affect the rate of successful germination.

Under cold stress conditions, the rate of seed water uptake lags, late embryogenesis-abundant (LEA) proteins are induced, dehydrin gene expression can be induced in germinating seed, and higher levels of nuclear-localized growth-repressing proteins down-regulate growth (Yao et al. 2005; Achard et al. 2008). Cold-Binding Transcription Factor/DREB1 (a *cis*-acting dehydration-responsive element binding protein) transcriptional activators and calmodulin binding transcription activators are crucial in cold regulation of gene expression and subsequent freezing tolerance (Savitch et al. 2005; Doherty et al. 2009). *BN115*, a gene related to ABA-Insensitive3/Viviparous1 (*ABI3/VP1*) from *B. napus* cv. HuYou15, and cold responsive genes that encode LEA-like proteins in response to dehydration stress, such as *BnCOR25*, have been reported as low-temperature response genes in various *B. napus* cultivars (Sangwan et al. 2001; Chen et al. 2011; Zhuang et al. 2011).

Cold acclimation alters *B. napus* plant phenotype and growth (Savitch et al. 2005; Dahal et al. 2012). Rapid early growth under lower temperatures - or early vigour - accelerates the rate of canopy development, radiation interception and increased transpiration resulting in increased dry matter (Gunasekera et al. 2006). The increased leaf thickness associated with the cold-acclimated state can be accounted for by either increases in leaf mesophyll cell size (Hüner et al. 1981; Gorsuch et al. 2010) or increases in the number of palisade layers (Boese and Hüner 1990; Dahal et al. 2012).

Low temperatures negatively affect yield by decreasing the number and size of seeds per silique (Morrison and Stewart 2002). However, cool temperatures (10 to 15 °C) have been noted to lead to higher oil content in *B. napus* seeds. Cold acclimation during development and germination is correlated with high levels of ABA, which is also correlated with high photosystem II (PSII) excitation pressure (Rapacz et al. 2003). Absciscic acid has high activity for induction of oleosin genes (Qi et al. 1998). The accumulation of oleosins results in relatively small oil bodies that are

typical of high oil content cultivars (Hu et al. 2009).

## **2.5 Abiotic stresses to *B. napus* growth: salt stress**

Approximately 275 Mha of the world's arable land is irrigated, >20 % of irrigated land is affected by salinity and approximately 37 % are sodic (Ghassemi et al. 1995; Chen and Jiang 2010). Additionally, marginal lands that used to not be cultivated because of a high degree of salinity are now being cultivated, owing to the increasing global demand for food. The major contributors to increasing salinity include low precipitation and the deposition of oceanic salts carried in wind and rain, high surface evaporation, and the weathering of rocks that release soluble salts of various types, particularly sodium chloride, which is the most soluble and widespread salt. The poor quality of water used for irrigation can exacerbate the salinization of once productive agricultural lands (Ashraf and Foolad 2007).

*Brassica napus* is the most salt tolerant of the brassicaceous crops, but salt stress inhibits *B. napus* growth and the production and quality of the crop are greatly reduced by soil salinity (Ruiz and Blumwald 2001; Akbari et al. 2011). Salt causes osmotic, ionic, and oxidative stresses in *B. napus* plants. Osmotic stress involves the reduction of the water potential outside the roots. It affects plant growth immediately, and is visible primarily as it becomes difficult for plant roots to take up water from the soil, and the growth of new shoots decreases (reviewed by Munns and Tester 2008). The *B. napus* leaf water potential has been observed to decrease in response to  $200 \times 10^{-3}$  M NaCl treatment (e.g., Akbari et al. 2011). Akbari et al. (2011) also found that the proline content increased in NaCl-treated *B. napus* leaves. Proline is known to be associated with osmotic tolerance (reviewed by Chen and Jiang 2010). A 22 kD polypeptide with a possible protective role against drought stress (BnD22) was observed in *B. napus* leaves subjected to  $0.4 \times 10^{-3}$  M NaCl (Reviron et al. 1992).

One of the most dramatic and readily measurable responses induced by the osmotic effect of salt is a decrease of stomatal aperture, which consequently limits CO<sub>2</sub> uptake (Halliwell 1987). Stress-induced stomatal closure reduces the leaf internal CO<sub>2</sub> concentration, diminishes carbamylation, and promotes dead-end binding of ribulose-1,5-bisphosphate to the active sites of RuBisCO.

The shoots and roots of *B. napus* plants treated with sodium chloride accumulate and subsequently redistribute sodium ions throughout the plant (Munns and Tester 2008). The xylem imports sodium ions to the leaf blade, where they inhibit enzyme activity and disturb metabolism (Munns and Tester 2008). As such, the increased senescence of older leaves is the primary visible effect of ionic stress. In the shoots and roots, ion accumulation coincides with the upregulation of membrane proteins such as voltage-dependent anion-selective channel protein, porin-like protein, and vacuolar ATPase (Cheng et al. 2012).

Salt stress impairs the electron transport chain and increases the formation of reactive oxygen species - cellular indicators and secondary messengers of stress - such as superoxide radicals ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals (Sharma and Dietz 2009; Cheng et al. 2012). Reactive oxygen species damage the plant by oxidizing photosynthetic pigments, membrane lipids, proteins and nucleic acids (Reddy et al. 2004, Pang and Wang 2008). An end product of membrane lipid peroxidation, malondialdehyde, increased in *B. napus* plants stressed with  $200 \times 10^{-3}$  M NaCl, compared to controls (Akbari et al. 2011). Chlorophyll a and b, and total soluble protein, content have been observed to decrease, owing to the destruction of chlorophyll pigments and the instability of pigment complexes (Ashraf and McNeilly 2004; Athar et al. 2009).

The major effect of salinity in dicotyledonous species is the dramatic curtailing of the size of individual leaves, the numbers of branches, and leaf photosynthesis (Munns and Tester 2008; review by Parida and Das 2005). Bilgili et al. (2011) found the number of leaves produced by brassicaceous species was inversely related to the salt concentration of the growing media. Smaller plants may be less vulnerable to stress because they have less surface area. Salt stress has been shown to reduce GA and elevate ethylene, mediators of almost every aspect of plant growth and development, *e.g.*, thereby enhancing DELLA restraint of plant cell proliferation and expansion (Achard et al. 2006; Mayak et al. 2004). The above- and below-ground fresh and dry weights of *B. napus* plants decrease as the salt concentration of the growing medium increases from 50 to  $200 \times 10^{-3}$  M NaCl (Sergeeva et al. 2006). Similarly, Bilgili et al. (2011) found that the number of leaves of 45-day-old *B. napus* cv. Bristol seedlings was inversely related to the salt concentration of the growing medium; as the salt concentration increased from 0 to  $100 \times 10^{-3}$  M

NaCl leaf number decreased by 24 %. Bybordi et al. (2010) observed that even  $50 \times 10^{-3}$  M NaCl caused *B. napus* cv. SLM<sub>046</sub> dry biomass production to decrease somewhat, and 100 and  $150 \times 10^{-3}$  M NaCl reduced biomass production by 25 and 35 %, respectively (Bybordi et al. 2010). Bybordi et al. (2010) observed a 55 % decrease in dry biomass at  $200 \times 10^{-3}$  M NaCl. Cheng et al.'s (2012) proteome analysis found 19 protein spots involved in the down-regulation of photosynthesis in response to salt stress in *B. napus* shoots but not roots.

The activities of antioxidant enzymes such as peroxidase, superoxide dismutase, and catalase have been observed to increase in salt stressed *B. napus* seedlings (Akbari et al. 2011). These antioxidant enzymes are important in the scavenging system of reactive oxygen species (Li 2008). In *B. napus* leaves stressed with salt, Akbari et al. (2011) found low-molecular weight antioxidant ascorbic acid, in its reduced and oxidized forms, and glutathione levels increased, and its oxidized form decreased. Cheng et al. (2012) found monodehydroascorbate reductase upregulated, which is crucial for the regeneration of a major antioxidant, ascorbate.

## 2.6 Natural plant growth regulators

The initiation and normal development of floral buds of *Brassica napus*, *in vitro*, from sepal primordia stage to maturity, is dependent on the presence of cytokinin, and is sensitive to the initial pH of the growth medium (Polowick and Sawhney 1991). Benzylaminopurine was more effective than other cytokinins (kinetin, zeatin, and zeatin riboside) for the *in vitro* stimulation of the initiation of additional buds on the *B. napus* inflorescence axis.

Gibberellins applied at  $10^{-3}$  to  $10^{-1}$  M to winter cv. Crystal promoted stem elongation:  $GA_3 > GA_1 > GA_4 > GA_5$  (Zanewich 1993). And  $GA_3$  induced parthenocarpy and cellular expansion in mesocarp tissues (Srinivasan and Morgan 1996). By degrading DELLA protein, promoting auxin synthesis, increasing the length of hypocotyls, and increasing fresh weight, gibberellins can protect *B. juncea* from severe salt stress and *B. campestris* seedlings from osmotic stress and *B. napus* plants from cadmium stress ( $100 \times 10^{-6}$  M  $Cd^{2+}$ ) (Fisah and Höfner 1995; Meng et al. 2009).

Exogenously applied  $12.5 \times 10^{-6}$  M jasmonic acid (JA) alleviated cadmium stress for 34-day-old *B. napus* cv. Zhe-Shuang 72 plants. Under moderate stress conditions ( $50 \times 10^{-6}$  M  $Cd^{2+}$ ), the

JA-treated plants had higher fresh weights; contained less malondialdehyde, representing less membrane lipid peroxidation; and had significantly increased chlorophyll concentrations (+ 61.4 %), compared to the controls (Meng et al. 2009). The application of JA reduced the activities of antioxidant enzymes ascorbate peroxidase, superoxide dismutase, peroxidase and catalase in the Cd-stressed plants (Meng et al. 2009).

### **2.6.1 Natural plant growth regulator: abscisic acid**

The hormone ABA has been shown to be involved in plant responses to high temperature, water stress, and infection, *e.g.*, with the hemibiotrophic ascomycete *Leptosphaeria maculans* (Qaderi et al. 2012, Šašek et al. 2012). Absciscic acid balances transpiration by affecting stomatal conductance, it triggers stomatal closure, it induces and maintains seed dormancy, and it regulates germination in response to internal and external factors. However, reduced ABA content of seeds under higher temperatures has also been reported.

Hays et al. (2007) found that, specifically with respect to *B. napus* cv. Topas zygotic embryos, the transition to the dry seed stage was accompanied by a reduction in ABA, which agrees with the paradigm of *B. napus* producing nondormant seed. Schopfer and Plachy (1984) found physiologically insignificant levels of endogenous ABA in mature *B. napus* seed. Similarly, the seeds of ABA-deficient and ABA-insensitive Arabidopsis mutants exhibit reduced dormancy (Karssen et al. 1983; Koornneef et al. 1984). During Arabidopsis seed development and germination 9-*cis*-epoxycarotenoid dioxygenase genes *NCED6* and *NCED9* are the major genes responsible for ABA synthesis; and constitutive expression of a 8'-hydroxylase gene (*CYP707A*) resulted in decreased ABA content in mature dry seeds and a shorter after-ripening period to overcome dormancy (Lefebvre et al. 2006; Millar et al. 2006).

The balance of ABA and GA, and the concentration of ABA in imbibed seeds, as opposed to dry seed, are factors that determine germination (Millar et al. 2006, Zhang et al. 2010). Endogenous and external signals can strongly influence hormone levels (Ali-Rachedi et al. 2004; Millar et al. 2006). Inhibitors of ABA biosynthesis, such as fluridone or norflurazon, enhance seed germination, and after-ripening, darkness, smoke, and stratification have been used to break

dormancy and have been strongly correlated with a decrease of ABA in seeds (Debeaujon and Koornneef 2000; Gubler et al. 2005).

Although treatment with ABA, or increased sensitivity to ABA concentrations, can produce dormancy in brassicaceous seed, priming *B. napus* seed with  $100 \times 10^{-6}$  M ABA reduced the transcript levels for peroxiredoxin, and induced the expression of calmodulin and aquaporins, and triggered rapid and uniform germination and increased the final percent of the seed that germinated under  $100 \times 10^{-3}$  M NaCl conditions, at 8 °C, compared to controls (Zheng et al. 1998; Gao et al. 2002). However, priming with ABA for an insufficient time resulted in no effect on seed germination (Gao et al. 2002). An ABA solution at  $10^{-6}$  M concentration reduced the hypocotyl lengths of both *B. napus* cv. Westar and cytoplasmic male sterile *B. napus*, whereas  $10^{-5}$  M ABA was totally inhibitory (Singh and Sawhney 1992).

Most of the known hormone families have been shown to influence shoot branching in some way. Absciscic acid inhibits bud growth (Chatfield et al. 2000). Basally applied ABA can enhance apical auxin-mediated Arabidopsis bud inhibition and apically applied ABA can reduce it (Chatfield et al. 2000). Tissue age was found to be an important factor in the effects of ABA on apical dominance, at least with respect to runner beans (*Phaseolus coccineus* [L.] cv. Weiszer Ries) (Hartung and Fiinfer 1981).

Winter genotype ‘Zhe-Shuang 72’ plants that were Cd-stressed and treated exogenously with  $10 \times 10^{-6}$  M ABA had higher fresh weights, less malondialdehyde concentration in leaves, and more leaf chlorophyll (+67.1 %), compared to controls (Meng et al. 2009). The ABA-treated plants had lower internal Cd accumulation at the 5–7 leaf stage, at  $100 \times 10^{-6}$  M Cd<sup>2+</sup>, compared to controls (Meng et al. 2009). The ABA treatment decreased ascorbate peroxidase and superoxide dismutase activities of plants grown under 50 or  $100 \times 10^{-6}$  M Cd<sup>2+</sup> conditions, and decreased peroxidase activity at  $100 \times 10^{-6}$  M Cd<sup>2+</sup> and catalase activity at  $50 \times 10^{-6}$  M Cd<sup>2+</sup>.

### **2.6.2 Natural plant growth regulator: salicylic acid**

Salicylic acid (SA) mediates inducible plant defenses (Potlakayala 2006). The physiological role of SA is as a signal molecule. The interaction between the SA- and JA/ethylene-dependent signaling pathways appears to be complex. Mutual antagonism is the primary mode of interaction (Feys and Parker 2000). Beckers and Spoel (2006) speculated that, upon

encountering multiple signals, inter-pathway communication fine-tunes and prioritizes plant defense responses. Depending on concentration, and the method and time of application, salicylic acid-dependent signal transduction pathways can: affect resistance to certain pathogens, *i.e.*, biotrophs that derive energy from living host cells; affect the rate of generation of reactive oxygen species under stress; alter the activities of antioxidant enzymes; and increase plant tolerance to abiotic stresses (Glazebrook 2005; reviewed by Horvath et al. 2007).

Treatment with SA induced H<sub>2</sub>O<sub>2</sub> generation in *Arabidopsis*, but in *B. napus*, treatment with SA can elevate antioxidant enzymes and reduce O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, and malondialdehyde levels in leaves, leading to alleviation of the oxidative damage (Kazemi et al. 2010; Cui et al. 2010). In nickel-stressed *B. napus* plants and *B. napus* plants exposed to napropamide herbicide, salicylic acid treatments reduced H<sub>2</sub>O<sub>2</sub> accumulation (Rao et al. 1997; Kazemi et al. 2010). Also in *B. napus* exposed to napropamide, salicylic acid treatment decreased the activities of superoxide dismutase, catalase, and ascorbate peroxidase, and increased activities of guaiacol peroxidase and glutathione-S-transferase (Cui et al. 2010).

Salicylic acid plays an important role in the regulation of growth and participates in the stabilization of cell membranes (reviewed by Raskin 1992). Treatment with SA alone increased the above ground dry weight of *B. napus* cv. PF plants (Kazemi et al. 2010). Cui et al. (2010) improved *B. napus* growth with SA treatment, and reduced levels of plant napropamide herbicide content from a contaminated environment.

In contrast to *Arabidopsis*, where SA signaling is not required for resistance, *Brassica napus* defenses against biotrophic invasion, *e.g.*, by *L. maculans*, are regulated mainly by SA (Oliver and Ipcho 2004, Šašek et al. 2012). Salicylic acid has been known for many years to regulate local defense to pathogen attack, at the site of infection, and SA levels also increase as part of the systemic resistance response in uninfected tissues (reviewed by Ryals et al. 1996, Loake and Grant 2007). Inoculation of *B. napus* plants with avirulent *Pseudomonas syringae* pv. *maculicola* 1120B resulted in a 28-fold increase in SA levels in local tissues and a roughly twofold increase in leaves that were not inoculated directly (Potlakayala et al. 2007). Salicylic acid causes callose buildup in plasmodesmata in response to pathogen attack (Wang et al. 2013). Salicylic acid derivatives can be transported over long distances, principally via phloem or xylem (Ratzinger et al. 2009). High concentrations of SA occur in phloem sap after pathogen attack,

and concentrations of SA and its glucoside can increase in xylem sap from the root and hypocotyl of the plant, and in extracts of shoots above the hypocotyl after infection, *e.g.* with the vascular pathogen *Verticillium longisporum* (Ratzinger et al. 2009). Systemic acquired resistance (SAR) in *B. napus* displays many of the hallmarks of classical SAR including long lasting and broad host range resistance, the activation of pathogenesis-related genes and proteins, *e.g.*, *BnPR-1* and *BnPR-2* (Durrant and Dong 2004, Potlakayala et al. 2007). Treatment of *B. napus* with menadione sodium bisulphate seems to induce a form of systemic resistance distinct from SAR, and it enhanced resistance to *L. maculans* (Borges et al. 2003). A soil drench of SA increased the concentration of glucosinolates, especially 2-phenylethylglucosinolate, in *B. napus* leaves, depending on the concentration of SA applied, and the age of the leaf (Kiddle et al. 1994).

Whereas the foliar application of  $10 \times 10^{-6}$  M SA can cause seed oil glucosinolate accumulation, after the imposition of drought stress it can reduce oil glucosinolates; increase proline accumulation; increase leaf soluble proteins; overcome the drought-induced decrease of seed oil oleic acid; and protect chlorophyll *a* and carotenoids for cvs. Rainbow and Dunkeld (Ullah et al. 2012). Exogenous application of SA ameliorates the damaging effects of heavy metals, such as cadmium, lead, mercury, and nickel (Kazemi et al. 2010). Treatment with  $50 \times 10^{-6}$  M SA, under moderate Cd stress ( $50 \times 10^{-6}$  M Cd<sup>2+</sup>) conditions, decreased the chlorophyll concentration of *B. napus* plants by 57.0 % (Meng et al. 2009). Salicylic acid can reduce root-to-shoot translocation of nickel and increased the activities of the antioxidant enzymes in leaves of nickel-stressed plants (Kazemi et al. 2010). Salicylic acid with chelating action on metals may decrease malondialdehyde content in plants under heavy metal stress (Horváth et al. 2007). Once established, systemic acquired resistance can last several weeks (Hammerschmidt and Kuć 1995). Systemic acquired resistance is also effective in field conditions as demonstrated in trials with bean, tobacco, and cucumber (Sutton 1982).

## 2.7 Synthetic plant growth regulators

The triazoles are the largest group of plant and animal disease-controlling compounds (Siegel 1981). They are fungitoxins and PGRs that require only low application rates (Davies et al. 1988, Fletcher et al. 1986). Triazoles affect GA anabolism and ABA catabolism, sterol biosynthesis, energy metabolism, photosynthesis, stress tolerance, and assimilate distribution (Ijaz 2012).

Triazoles affect the isoprenoid pathway, reduce ethylene, and increase cytokinin levels, and inhibit mono-oxygenases at cytochrome P450. Where the mono-oxygenase would oxidize *ent*-kaurene to kaurenoic acid, the triazole blocks GA synthesis (Ijaz 2012). Many triazoles persist in the soil long after use (Daniels and Scarisbrick 1986). Some triazole compounds, *e.g.*, tebuconazole, have been characterised as endocrine disruptors in mammals (Taxvig et al. 2007).

Paclobutrazol (PP333) is a triazole that blocks the enzyme kaurene oxidase that catalyzes the oxidation of *ent*-kaurene to *ent*-kaurenoic acid, thereby inhibiting GA synthesis (Rademacher et al. 1984). Low doses of PP333 can reduce shoot elongation, but high doses can prevent stem elongation and delay or inhibit flowering (Rood et al. 1987; 1989). Rao and Mendham (1991) found that PP333 treatment shortened *B. napus* cv. Marnoo and prevented lodging, but reduced yield. Applied as a root drench or foliar spray, paclobutrazol inhibited stem elongation, but only delayed the flowering of fully vernalized winter cv. Crystal (Zanewich 1993). Paclobutrazol contamination can affect non-target organisms (Wu et al. 2013).

By inhibiting GA and sterol synthesis, increasing water content, reducing water consumption, and increasing the CO<sub>2</sub> fixation rate, triapenthenol (RSW0411) treatment at the bud stage can reduce *B. napus* height and increase branching, total siliquae plant<sup>-1</sup>, and yield (Daniels and Scarisbrick 1986; Kirkland 1992). Triapenthenol decreases GA<sub>19</sub>, GA<sub>20</sub>, GA<sub>1</sub> and GA<sub>8</sub> content, which Fisah and Höfner (1995) correlated to stem height. However, the annual growing conditions, the dose, and the plant developmental stage of application are factors in the effectiveness of RSW0411 to increase lodging resistance and yield (Kirkland 1992).

The growth retardant trinexapac-ethyl (Syngenta product name Moddus) is a cyclohexanedione that inhibits GA production later in the biosynthetic pathway than triazoles (Rademacher 2000). The timing of trinexapac-ethyl application is flexible and it is more persistent, compared to PGRs from other chemical groups (Ijaz 2012). Mixing triazole and strobilurin fungicides with trinexapac-ethyl enhances the anti-lodging effect in winter varieties (Ijaz 2012).

Trinexapac-ethyl, which has also been known as CGA163'935, is ethyl 4-cyclopropyl (hydroxy) methylene-3,5-dioxocyclohexane-carboxylate, a plant growth retardant that takes action late in

the GA pathway by interacting with the 2-oxoglutarate binding site of dioxygenases, cofactors of some biosynthetic GA enzymes including 3 $\beta$ -hydroxylase which catalyzes the formation of GA<sub>1</sub> from GA<sub>20</sub> (Rademacher et al. 1992).

Timing is crucial for cerone (ethephon) application. Cerone generates ethylene and can reduce plant height and significantly increase yield (Jones 1983). However, yields were either similar or reduced, particularly for applications to the Bienvenu cultivar during early stem elongation at two levels of N (Daniels and Scarisbrick 1986). Only application at the start of flowering increased yield by 10 % and did not produce secondary flowering. Cerone can cause abortion of entire racemes, particularly if applied at the green bud stage (Daniels and Scarisbrick 1986). BASF's plant protection product Terpal contains an antigibberellin, mepiquat chloride, and cerone (Waring 1980). It consistently reduced *B. napus* plant height in field trials (Daniels and Scarisbrick 1986). Gendy and Marquard (1989) found that a spray of 6 L ha<sup>-1</sup> tebepas (280 g L<sup>-1</sup> 2,3-Dichloroisobutyric acid + 134 g L<sup>-1</sup> chlor-cholin-chloride + 55 g L<sup>-1</sup> cerone) decreased *B. napus* cv. Arabella stem height, but increased branch number, buds plant<sup>-1</sup>, seed yield, 1000-seed weight, and seed oil content.

High doses of chlormequat inhibited 33-day-old *B. napus* plant stem elongation; they remained as rosettes (Rood et al. 1987). Intermediate doses delayed flower development and inhibited stem elongation. Other groups found only transitory reductions in growth rate immediately following application (Scarisbrick et al. 1985; Daniels and Scarisbrick 1986). In Czechoslovakia, spring applications of 4-6 kg chlormequat ha<sup>-1</sup> to winter cvs. increased yields, but Daniels and Scarisbrick (1986) saw consistent increases only for Rafal in 1981-82 and Jet Neuf in 1982-83.

Under control (not drought) conditions, a putrescine spray lowered the chlorophyll *a/b* and seed oil/protein ratios of *B. napus*, but lifted the seed oil oleic/linoleic acid ratio (Ullah et al. 2012). It decreased glucosinolates (13 %) for cv. Dunkeld. Under drought conditions, it increased carotenoids, proline, seed oil oleic acid, chlorophyll *a/b* ratio of *B. napus* plants, and it increased the oil content of Rainbow seed (Ullah et al. 2012). Under drought conditions, putrescine treatment increased seed oil glucosinolate (52 %) and erucic acid in Dunkeld.

The relatively hydrophobic auxin analogue naphthaleneacetic acid (NAA) may diffuse more freely into cells, and can enter cells, without an auxin influx carrier (Delbarre et al. 1996). The IAA receptor TIR1 is able to bind to NAA. Like 2,4-Dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid acts on auxin signaling and affects the metabolism of cytokinins, in part through the activity of cytokinin oxidases (Palni et al. 1988). Julliard et al. (1992) found faster *B. napus* root regeneration and reduced shoot organogenesis due to NAA. Naphthyl acetic acid applied to emasculated flowers produced abnormal pods by expanding the mesocarp parenchyma (Srinivasan and Morgan 1996).

Keiller and Morgan (1988) reported a longer flowering stage and longer stems due to the application of benzyladenine (BA), but it had no effect when applied 14 d after anthesis or later. Application of BA at 1-5 rather than at 11-15 days after flower opening led to the development of bigger pods (Srinivasan and Morgan 1996). Treatments of BA to emasculated flowers induced parthenocarpy, but BA treatment to non-emasculated flowers on the terminal raceme delayed senescence and increased the size of apical pods (Srinivasan and Morgan 1996).

Daniels and Scarisbrick (1986) reported variability due to the season of application for the plant growth regulator R201, which they applied to reduce height and increase lodging resistance. The first two spring treatments reduced the length of all internodes. The autumn application shortened only the lower internodes, the upper ones grew longer than the controls.

Most PGRs currently applied to *B. napus* in agriculture are plant growth retardants. Many are endocrine disruptors, neurotoxic, and persistent in the soil (Daniels and Scarisbrick 1986; Taxvig et al. 2007). The PGRs that are hormone analogues can have unintended effects on the health of nontarget organisms in the agroecosystem (Wu et al. 2013). In light of these circumstances, new PGRs such as the signal molecules investigated here, which have shown no negative health side effects thus far, may provide alternatives. Awareness of the response of plants to these signal molecules will help us understand their potential as plant growth regulators. If they are effective in this capacity this will have the potential to diversify the tools available to producers for the optimal management of their crops.

## 2.8 Chitopentaose

Chitopentaose is a linear polysaccharide composed of  $\beta$ -N-acetylglucosaminyl residues (GlcNAcs) derived from chitin. Chitin is present in the exoskeleton of arthropods, fungal cell walls, and yeast (Rinaudo 2006). Plants often detect its presence and respond as they would to the presence of a pathogen or herbivore (Barber 1989; Yamada et al. 1993).

Five putative lysin motif (LysM)-containing chitin elicitor receptor-like kinase (RLK) genes have been identified in *Arabidopsis*, including RLK1, also known as chitin elicitor receptor kinase 1 (CERK1), which induces plant defense after binding to penta- to octameric chitin more strongly than chitin oligomers (Petutschnig et al. 2010; Iizasa et al. 2010). At  $10^{-8}$  M chitopentaose triggered maximal  $\text{Ca}^{2+}$  spiking, upon which the rhizobial and arbuscular mycorrhizal (AM) host signal transduction pathways relies, in barrel medic (*Medicago truncatula* [Gaertn.]) root organ culture atrichoblast cells (Genre et al. 2013). However, inactivation of the *M. truncatula* Nod factor perception gene, which encodes LysM RLK, did not modify the  $\text{Ca}^{2+}$  spiking activity of short-chain chitin oligomers (Arrighi et al. 2006).

Chito-oligosaccharides may be perceived by plants as microbial-associated molecular patterns. For example, the root-hair deforming active fraction produced by the nitrogen fixing *Frankia*, which is the symbiont of actinorhizal plants, contains GlcNAc (C  r  monie et al. 1999). Microbial-associated molecular patterns can lead to pattern-triggered immunity (Hamel and Beaudoin 2010). Pattern-triggered immunity would typically involve such processes as the accumulation of reactive oxygen intermediates, deposition of callose, large-scale transcriptional reprogramming, and biosynthesis of microstatic and/or microcidal secondary metabolites (Belkhadir et al. 2012). Chito-oligosaccharides elicit plant defense reactions in relatively diverse plants, as compared to Nod factor recognition, which is thought to be highly specific (Wan et al. 2008). For example, chitin fragments can elicit defense responses such as phytoalexin synthesis in rice (*Oryza sativa* [L.]) cultures (Yamada et al. 1993). Chitopentaose, at a concentration of  $4.0 \text{ mg mL}^{-1}$ , elicited lignification in wheat (*Triticum aestivum* [L.]) leaves wounded 16 h before inoculation (Barber et al. 1989).

Wang et al. (2010) produced chitopentaose, among other things, after five days of fermentation of squid pen biowaste with *Pseudomonas aeruginosa* K187 that was isolated from soil in Taiwan. The resultant culture supernatant enhanced Chinese cabbage (*Brassica chinensis* [L.])

growth. Wang et al. (2010) attributed the plant growth enhancement to amino acids, peptides, and *N*-acetyl chitooligosaccharides from the hydrolysis of the squid pen powder by protease and chitinase. Furthermore, chitosan has been shown to promote growth of radish (*Raphanus sativus* [L.]) (Tsugita et al. 1993 cited in Ohta et al. 2000 [in Japanese]) and enhance the germination of *T. aestivum* and maize (*Zea mays* [L.]) (Guan et al. 2009).

## 2.9 Lipo-chitooligosaccharide (LCO)

Rhizobium-legume, rhizobium-*Parasponia*, and AM symbiotic interactions are mediated by LCOs. For most legumes, lipo-chitooligosaccharide signals play a pivotal role for host-symbiont specificity and the induction of the early steps of infection and root nodule organogenesis, including symbiotic gene activation leading to mitotic reactivation of the cortical cells, and the formation of pre-infection threads (Oldroyd et al. 2010; Op den Camp et al. 2011). The synthesis of the chitin oligomer backbone requires the activity of three specific enzymes, encoded by the *nodABC* genes, which are present in almost all rhizobia characterized thus far, and can be found in symbiotic plasmids or islands that may be mobilized by lateral transfer among rhizobia (Rogel et al. 2001, Sullivan and Ronson 1998). Certain species of *Bradyrhizobium* do not contain *nod* genes within their genome, and they can induce nodulation without Nod factor signaling (Giraud et al. 2007). A single species of *Bradyrhizobium* can produce several unique LCOs, also called Nod factors (Carlson et al. 1993). Since Lerouge et al. (1990) first described the structure of a nodulation signal molecule produced by *Rhizobium meliloti*, many other LCOs have been structurally identified. The AM fungus *Glomus intraradices* also secretes symbiotic sulphated and non-sulphated simple LCOs, which stimulate the formation of AM symbiosis in plant species of diverse families: *Fabaceae*, *Asteraceae*, and *Umbelliferae* (Maillet et al. 2011). Preliminary analysis suggested that the uncultured Dg1 symbiont of Durango root (*Datisca glomerata* [C. Presl] Baill.) contains *nodABC*-like genes, which suggests that it uses Nod factor-like compounds during the infection process (Persson et al. 2011).

The Nod factor we applied was Nod Bj V (C18:1, MeFuc), which is produced by *Bradyrhizobium japonicum* 532C. It is 1415 Da and is, therefore, not a large molecule (Stacey et al. 1995). It is a fatty acylated chitin pentasaccharide, *i.e.*, five GlcNAc residues with a 2-*O*-methyl-fucose residue attached to the 6-position of the reducing-end GlcNAc. The fucosylation

of Nod factors by *B. japonicum* has been linked to nodulation of siratro (*Macroptilium atropurpureum* [DC.] Urb.) and ricebean (*Vigna umbellata* [Thunb.] Ohwi and Ohashi) but is not necessary for nodule organogenesis in wild soybean (*Glycine soja* [Siebold and Zucc.]) (Sanjuan et al. 1992; Stacey et al. 1994). It is likely that the lipid decoration of the LCO molecule is involved in the specificity of perception (Gough and Cullimore 2011). The relative orientation of the oligosaccharide and acyl moieties can range from being extended to essentially perpendicular to being quasi-parallel (Groves et al. 2005; Morando et al. 2011).

At millimolar concentrations, lipo-chitooligosaccharides form large molecular weight aggregates in aqueous solution, but it is likely monomeric at physiological concentrations (Groves et al. 2005). It is accumulated with relatively low levels of uptake, concentrated, and immobilized by binding sites in the plant cell wall, which become saturated by micromolar concentrations, before interaction with any receptors, such as LysM RLKs in the plasma membrane (Goedhart et al. 2000). Lysin motif RLKs are critical for the perception of Nod factors and the legume–rhizobial symbiotic interaction (Radutoiu et al. 2003). Ohnuma et al. (2008) showed that the binding affinities of the LysM domains increase as the number of GlcNAc oligomer repeats increases.

As a member of Brassicaceae, *Brassica napus* does not have any specialized root adaptations for nutrient acquisition and has neither symbiotic nor mycorrhizal associations (Ocampo et al. 1980). However, plants that do not host rhizobia may nevertheless perceive LCO. Ultra-performance liquid chromatography – electron spray ionization quantification has shown that LCO can increase total ABA (10.19 %) and salicylic acid (15.00 %), and decrease indolylacetic acid (-49.68 %), cytokinin (-36.24), gibberellic acid (-19.41), and JA (-33.66) of *Arabidopsis* rosettes, 24 h post treatment (Subramanian 2014). These levels of SA accumulation are somewhat less than the 100 to 200 % increase over background levels that would be expected to be induced at the primary leaf due to second stage systemic acquired resistance (SAR) (Cameron et al. 1999). While SAR is associated with an increase in transcript levels of pathogenesis-related genes, pathogen response protein 1 (PR1) was not found to increase in LCO-treated *Arabidopsis* (Subramanian 2014). However, lipo-chitooligosaccharides can have mitogenic and morphogenic effects similar to cytokinins and inhibitors of auxin transport (Dénarié et al. 1996). At micromolar concentrations, lipo-chitooligosaccharides induce the expression of the isoflavone reductase gene, a marker gene of the isoflavonoid synthesis pathway (Savouré et al. 1994). This

response can also be induced by exogenously applied chitotetraose, even in nanomolar concentrations. Previous work has shown that Nod Bj V (C18:1, MeFuc) from *B. japonicum* USDA110 can stimulate cell division of *G. soja* and *M. atropurpureum* at  $10^{-10}$  M (Sanjuan et al. 1992). Nod Bj V (C18:1, MeFuc) produced by *B. japonicum* 532C may play a role in seed germination and the growth of diverse plants (Prithiviraj et al. 2003). Lipo-chitooligosaccharides from different rhizobia added to tomato (*Lycopersicon esculentum* [Mill.]) cell culture suspensions at  $10^{-8}$  M concentrations stimulated a rapid transient alkalization of the medium (Staehelin et al. 1994). This response is also induced by chitin fragments at lower concentrations (Felix et al. 1993), which indicates that this perception system is characteristic for chitin fragments or their derivatives rather than for LCOs. In any case, treatment with elicitor molecules changes membrane permeability, increases influx of  $H^+$  and  $Ca^{++}$ , and increases efflux of  $K^+$  (Mathieu et al. 1991; Scheel and Parker 1990). Hebe et al. (1999) noted efflux of  $Cl^-$  and  $K^+$  and influx of  $Ca^{2+}$  in spruce (*Picea abies* L.) cells elicited with GlcNAc oligomers. These symptoms precede defence responses and have been implicated in the signal chains mediating defence response induction (Boller 1989; Dixon and Lamb 1990).

## 2.10 Thuricin 17

The genus *Bacillus* is characterized by rod shaped, facultative aerobe, endospore forming bacteria that live in soil and often colonize the plant rhizosphere (Sarosh et al. 2009). Various strains of *Bacillus thuringiensis* produce bacteriocins. Bacteriocins are peptides, encoded by structural genes by members of the domain *Bacteria*, with bactericidal or static effects against bacteria closely related to the producer strain (Mabood et al. 2008). Gram-negative bacteria are protected from bacteriocins, and most other compounds of molecular weight above 600 Da, by their outer membrane, which prevents relatively large molecules from reaching the plasma membrane.

*Bacillus thuringiensis* non-*Bradyrhizobium* endophytic bacterium (BtNEB17) produces and excretes a bacteriocin known as thuricin 17 (MW 3137.0). Sequences for the peptide were published by Lee et al. (2009). Thuricin 17 has bactericidal and bacteristatic effects on *B. cereus* ATCC 14579, but does not inhibit *Bacillus subtilis* (Gray et al. 2006b). Both thuricin 17 and bacthuricin F4, which is produced by *B. thuringiensis* ssp. *kurstaki* BUPM4, weigh approximately 3 kDa, have similar N terminus sequences, and have similar spectra of

antimicrobial activities; thus they have both structural and functional similarities (Gray et al. 2006a, b; Kamoun et al. 2005). Therefore, a new grouping, class IId, was proposed to accommodate them (Mabood et al. 2008). Class IId bacteriocins produced by *Bacillus* strains are highly effective inducers of plant defense-related enzymes phenylalanine ammonia lyase, guaiacol peroxidase, ascorbate peroxidase, superoxide dismutase, and polyphenol oxidase (Jung et al. 2011).

Previous work has shown that thuricin 17 can enhance soybean (*Glycine max* [L.] Merr.) and corn (*Z. mays* L.) growth (Lee et al. 2009). Although thuricin 17 has not been confirmed as a signal in the phloem, peptides such as thuricin 17 could generate or amplify messages, and act as signals themselves within the phloem of *B. napus* (Giavalisco et al. 2006). De Freitas et al. (1997) inoculated *B. napus* with a variety of *Bacillus* species. The most effective *Bacillus* inoculant in their experiment was a *B. thuringiensis* isolate that significantly increased the number of siliquae by 30 to 54 %, in the absence of rock phosphate. De Freitas et al. (1997) attributed the effects they observed to the bacterial production of plant growth hormones.

### 3 Preface to Section 3

Section 3 is a manuscript by Schwinghamer, Souleimanov, Dutilleul, and Smith that has been submitted to *Environmental and Experimental Botany*. The literature cited in this section is listed in the reference section (page 160).

In this section, to set the stage for more specific assessments in the following section, we addressed the question of whether or not the response to lipo-chitooligosaccharide, thuricin 17, and chitopentaose by *B. napus* cultivars 02C3, 02C6, 04C111, and 04C204 had been modified as compared to Polo and Topas. The differences in the characteristics of germination due to the temperature conditions, the cultivar, the signal, and the signal concentration are presented. Each figure caption, figure, and table is presented at the end of the section.

This thesis consists of 4 papers. This is the first, which focuses on the beginning of development, *i.e.*, germination. The second focuses on subsequent growth and early development; the third focuses on development right through to yield, but using a rapid system; and the fourth focus on development, including plant architecture through to yield with conventional genotypes.

## **Novel plant growth regulators lipo-chitooligosaccharide (LCO), thuricin 17, and chitopentaose can enhance the germination of canola (*Brassica napus* [L.]) cultivars**

### **3.1 Abstract**

In the agricultural environments where canola (*Brassica napus*) is grown, slow germination can increase the susceptibility of *B. napus* seedlings to soil borne pathogens, decrease the vigor of young plants, delay maturity, and decrease yield. Bacterial products that enhance germination have been pursued and identified for a variety of plants. Three signal molecules were investigated: *Bradyrhizobium japonicum* 532C product lipo-chitooligosaccharide (LCO), *Bacillus thuringiensis* NEB17 product thuricin 17, and chitopentaose, which is a pentameric chitin chain and the backbone of the LCO molecule. Concentrations of LCO and thuricin 17 that increase the early germination of *B. napus* cv. Polo seed in Petri dishes, under controlled and standard temperature conditions, were identified. To determine the parameters of seed germination, and to determine whether or not the interaction effects of cultivar-by-temperature and signal-by-temperature have significant effects, Gompertz functions were estimated for six cultivars (02C3, 02C6, 04C111, 04C204, Polo, and Topas), which were supplied with LCO, thuricin 17, and chitopentaose in solution. The germination of Polo seed, that was treated with a  $10^{-6}$  M concentration of LCO, increased by 75.0 % in the 5 to 15 growing degree day period, compared to the controls. Such fast and early *B. napus* germination can, under field conditions, lead to increased emergence, canopy coverage, and yield. Further experimentation with other cultivars and a range of temperature conditions did not discern any other particular differences, following treatment with LCO, thuricin, or chitopentaose, under mid-range (24 h 20 °C) and ideal (24 h 25 °C) growing conditions. However, thuricin 17 and chitopentaose enhanced the germination of the 6 experimental cultivars, but their effects depended on the temperature. Thuricin 17 positively affected *B. napus* growth under abiotic stress conditions: treatment with  $10^{-11}$  M thuricin 17 solution increased germination for all 6 cultivars by 6.9 %, under 24 h 10 °C temperature conditions. Treatment with  $10^{-6}$  M chitopentaose solution increased germination for all 6 cultivars by 17.2 %, under 24 h 30 °C temperature conditions. The take home message is that the response by *B. napus* to thuricin 17 and chitopentaose can enhance germination under stressful temperature conditions, but with respect to LCO, the response was exclusive to Polo.

### 3.2 Introduction

Under typical agricultural field conditions, early germination is an important factor that affects the yield of annual varieties of canola (*Brassica napus* [L.]) in the northern temperate area of western Canada. Owing to a very short growing season, particularly in the prairie regions, *Brassica napus* is usually seeded in the early spring. At that time, temperature is below optimum for germination. Early spring seeding is necessary for the crop to reach maturity without experiencing reductions in yield due to summer heat stress. However, cool spring conditions can delay germination and emergence of spring sown *B. napus* by 1 to 2 weeks. Furthermore, *Brassica napus* must be planted relatively deep to establish successfully under dry soil surface conditions (Harker et al. 2012a). Owing to these and other factors, only 50 % of planted *B. napus* seed typically emerge under agricultural conditions (Harker et al. 2003). Reduced germination rates can increase the susceptibility of seedlings to soil borne pathogens, *e.g.*, phoma stem canker (blackleg) (*Leptosphaeria maculans*), decrease the vigor of young plants, delay maturity, and delay the depletion of red light by the crop canopy, which would otherwise inhibit weed seed germination (Silvertown 1980; Médiène et al. 2011).

Although science has known *B. napus* since the nineteenth century, systematic research on *B. napus* only began in the late 1930s (Juska and Busch 1994). At that time, many countries developed national policies to promote self-sufficiency in production of fats and oils. In the early 1970s, responding to health concerns, Canadian breeders produced higher oil varieties, with less than two percent erucic acid in the oil, and less than  $30 \times 10^{-6}$  M of glucosinolates per gram of air-dried oil-free meal (Statistics Canada 2013). In 1979, these Canadian low erucic acid varieties came to be known as canola or edible oilseed rape. The cultivated area of this crop has been expanding rapidly in Canada, in part owing to the intensifying demand for biodiesel feedstock (Franzaring et al. 2008). In 2000, the average yield in Canada was  $1.5 \text{ Mg ha}^{-1}$  (Statistics Canada 2012). From 2008 to 2010, the average yield in Canada was approximately  $1.9 \text{ Mg ha}^{-1}$  (Harker 2012b).

*Brassica napus* seed is able to germinate after a drying period or low temperature conditions, which is to say that *B. napus* produces orthodox nondormant dry seed. Dry *B. napus* seed requires only imbibition at a suitable temperature, *e.g.*,  $25^\circ\text{C}$ , for rapid germination (Kucera et al. 2005). Previous work has indicated that cool ( $\leq 10^\circ\text{C}$ ) temperatures delay germination but

neither damages the seed, nor affects the rate of successful germination (Nykiforuk and Johnson-Flanagan 1999). Under cold stress conditions, the rate of seed water uptake lags, late embryogenesis-abundant (LEA) proteins are induced, dehydrin gene expression can be induced in germinating seed, and higher levels of nuclear-localized growth-repressing proteins down-regulate growth (Yao et al. 2005; Achard et al. 2008). Cold-Binding Transcription Factor/DREB1 (a *cis*-acting dehydration-responsive element binding protein) transcriptional activators and calmodulin binding transcription activators are crucial in cold regulation of gene expression and subsequent freezing tolerance (Savitch et al. 2005; Doherty et al. 2009). *BN115*, a gene related to Absciscic Acid Insensitive3/Viviparous1 (*ABI3/VP1*) from *B. napus* cv. HuYou15, and cold responsive genes that encode LEA-like proteins in response to dehydration stress, such as *BnCOR25*, have been reported in various *B. napus* cultivars (Sangwan et al. 2001; Chen et al. 2011; Zhuang et al. 2011).

Rhizobia-legume and rhizobia-*Parasponia* symbiotic interactions are mediated by lipo-chitooligosaccharide signal molecules (reviewed by Oldroyd et al. 2010; Op den Camp et al. 2011). The arbuscular mycorrhizal (AM) fungus *Glomus intrradices* also secretes sulphated and nonsulphated simple LCOs that stimulate the formation of AM symbioses with plants in the *Fabaceae*, *Asteraceae*, and *Umbelliferae* families (Maillet et al. 2011). The Dg1 symbiont of Durango root (*Datisca glomerata* [C. Presl] Baill.) contains *nodABC*-like genes, which suggests that it uses Nod factor-like compounds during the infection process, too (Persson et al. 2011). For most legumes, lipo-chitooligosaccharides play a pivotal role in host-symbiont specificity and the induction of the early steps of infection and root nodule organogenesis, including symbiotic gene activation leading to mitotic reactivation of the cortical cells, and the formation of pre-infection threads (Oldroyd et al. 2010). The Nod factor we applied was Nod Bj V (C18:1, MeFuc) (MW 1415 Da). It is a fatty acylated chitin pentasaccharide, *i.e.*, five *N*-acetylglucosamine (GlcNAc) residues with a 2-*O*-methyl-L-fucosyl residue attached to the 6-position of the reducing-end GlcNAc. The relative orientation of the oligosaccharide and acyl moieties can range from being extended to essentially perpendicular to being quasi-parallel (Groves et al. 2005; Morando et al. 2011). It is likely that the lipid decoration of the LCO molecule is involved in the specificity of the perception of it by plants (Gough and Cullimore 2011). At  $10^{-3}$  M concentrations, lipo-chitooligosaccharides form large molecular weight aggregates in aqueous solution, but they are likely monomeric at physiological concentrations

(Groves et al. 2005). Lipo-chitooligosaccharide is accumulated with relatively low levels of uptake. It is concentrated and immobilized by binding sites in the plant cell wall, which become saturated by  $10^{-6}$  M concentrations, before interaction with any receptors, such as lysin motif (LysM)-containing chitin elicitor receptor kinases (RLKs) in the plasma membrane (Goedhart et al. 2000).

*Brassica napus* has neither symbiotic rhizobial nor mycorrhizal associations, but plants that do not host rhizobia may nevertheless perceive LCO. Five LysM RLK genes were identified in brassicaceous *Arabidopsis thaliana* (L.) Heynh., including RLK1, also known as CERK1, which induces plant defense after binding to penta- to octameric chitin more strongly than chitin oligomers (Petutschnig et al. 2010). Ohnuma et al. (2008) showed that the binding affinities of the LysM domains increase as the number of GlcNAc oligomer repeats increases. However, Liang et al. (2013) reported that non-legume plants, including *Arabidopsis*, recognized Nod Bj V (C18:1) via a mechanism that resulted in strong suppression of microbe-associated molecular pattern (MAMP)–triggered immunity, which lead to reduced levels of pattern-recognition receptors on plasma membranes involved in MAMP recognition. Ultra-performance liquid chromatography – electron spray ionization quantification has shown that treatment with Nod Bj V (C18:1, MeFuc) increased the total abscisic acid (10.19 %) and salicylic acid (15.00 %) contents, and decreased indolylacetic acid (-49.68 %), cytokinin (-36.24 %), gibberellic acid (-19.41 %), and jasmonic acid (-33.66 %) contents of *Arabidopsis* rosettes, 24 h post treatment (Subramanian, personal communication). These levels of salicylic acid (SA) accumulation are somewhat less than the 100 to 200 % increase over background levels that would be expected to be induced at the primary leaf due to second stage SAR (Cameron et al. 1999), and while SAR is associated with an increase in transcript levels of pathogenesis-related genes, pathogen response protein 1 (PR1) did not increase in LCO-treated *Arabidopsis* (Subramanian, personal communication). Previous work has shown that Nod Bj V(C18:1, MeFuc) from *B. japonicum* USDA110 can stimulate cell division of wild soybean (*Glycine soja* P1468397 [Siebold and Zucc.]) and siratro (*Macroptilium atropurpureum* [DC.] Urb.) at  $10^{-10}$  M (Sanjuan et al. 1992). Nod Bj V(C18:1, MeFuc) produced by *B. japonicum* 532C may play a role in seed germination and the growth of diverse plants (Prithiviraj et al. 2003). Lipo-chitooligosaccharides from different rhizobia added to tomato (*Lycopersicon esculentum* [Mill.]) cell culture suspensions at  $10^{-8}$  M concentrations stimulated a rapid transient alkalization of the medium (Staehelin et al.

1994). This response is also induced by chitin fragments at lower concentrations, which indicates that this perception system is characteristic for chitin fragments or their derivatives rather than for LCOs (Felix et al. 1993).

Chitopentaose is a linear polysaccharide composed of (1→4)-linked GlcNAc residues derived from chitin. Chitin is present in the exoskeleton of arthropods, fungal cell walls, and yeast (Rinaudo 2006). Chito-oligosaccharides may be perceived by plants as microbial-associated molecular patterns. Microbial-associated molecular patterns can lead to pattern-triggered immunity (Hamel and Beaudoin 2010). Chito-oligosaccharides elicit plant defense reactions in relatively diverse plants, as compared to Nod factor recognition, which is thought to be highly specific (Wan et al. 2008). For example,  $10^{-8}$  M chitopentaose, produced by glomeromycete *Rhizophagus irregularis*, triggered maximal  $\text{Ca}^{2+}$  spiking in barrel medic (*Medicago truncatula* [Gaertn.]) root organ culture atrichoblast cells (Genre et al. 2013). Wang et al. (2010) produced chitopentaose, among other things, after five days of fermentation of squid pen biowaste with *Pseudomonas aeruginosa* K187 that was isolated from soil in Taiwan. The resultant culture supernatant enhanced Chinese cabbage (*Brassica chinensis* [L.]) growth. Wang et al. (2010) attributed the plant growth enhancement to amino acids, peptides, and *N*-acetyl chitooligosaccharides from the hydrolysis of the squid pen powder by protease and chitinase.

*Bacillus thuringiensis* NEB17 produces and excretes thuricin 17. Thuricin 17 is a class IId bacteriocin with a broad range of antimicrobial activity against closely related bacterial species (Gray et al. 2006a). Sequences for the peptide were published by Lee et al. (2009). Although thuricin 17 has not been confirmed as a signal in the phloem, peptides such as thuricin 17 could generate or amplify messages, and act as signals themselves within the phloem of *B. napus* (Giavalisco et al. 2006). Class IId bacteriocins produced by *Bacillus* strains are highly effective inducers of plant defense-related enzymes phenylalanine ammonia lyase, guaiacol peroxidase, ascorbate peroxidase, superoxide dismutase, and polyphenol oxidase (Jung et al. 2011). Previous work has shown that thuricin 17 can enhance soybean (*Glycine max* [L.] Merr.) and corn (*Z. mays* L.) growth (Lee et al. 2009).

The broad objective of this work was to evaluate the potential of LCOs and thuricin 17 as plant growth regulators for use in the production of canola. The first specific objective was to determine the parameters of seed germination, which were estimated from Gompertz functions

for the cultivars of interest, which were supplied with LCO, thuricin 17, and chitopentaose in solution. The second objective was to determine whether or not the cultivar-by-temperature or signal-by-temperature interactions affected the Gompertz function parameters or the derived indices.

### **3.3 Materials and methods**

#### **3.3.1 Plant material**

The germination assay was conducted with open pollinated *B. napus* lines made using conventional crosses and pedigree (Dr. P. McVetty, University of Manitoba) (Table 3.1). The crosses that produced high erucic acid *B. napus* sister cvs. 04C111 and 04C204 are presented in standard breeder's notation, where '/' represents one cross, and increasing numbers of '/' marks indicates previous crosses, here: HiQ////Apollo///86LL141//Tatyoan/R83-14. The crosses that produced sister cvs. 02C3 and 02C6 are also presented in standard breeder's notation, here: Polo///86LL141//Tatyoan/R83-14. *Brassica napus* cv. Polo is a conventional, double low, *i.e.*, low glucosinolate and low erucic acid, spring cultivar. *Brassica napus* cv. Topas (SWAB, Svalöv, Sweden) is a commercial-quality cultivar. Topas was included initially in our experiments as a comparative control because of its relatively low seed oil content.

#### **3.3.2 Production of lipo-chitooligosaccharide**

*Bradyrhizobium japonicum* strain 532C was cultured in the Yeast Extract Mannitol (YEM) medium (Mannitol 10 g, K<sub>2</sub>HPO<sub>4</sub> 0.5 g, MgSO<sub>4</sub> 7•H<sub>2</sub>O 0.2 g, NaCl 0.1 g, yeast extract 0.4 g, distilled water 1 L) under continuous shaking on an orbital shaker (Model 5430 Table Top Orbital Shaker; Forma Scientific Inc., Marietta, OH, USA) at 150 rpm and 27 ± 2 °C. When the OD<sub>620</sub> reached 0.4 to 0.5 (4 to 5 days), genistein (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was added to a final concentration of 5 × 10<sup>-6</sup> M and the culture was incubated for an additional 48 h. The culture was extracted with 0.4 volume of HPLC grade 1-butanol by shaking vigorously for 10 to 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 (Yamota RE500; Yamato, San Francisco, CA, USA). The resulting material was dissolved in 20 % aqueous acetonitrile (ACN) and this constituted the LCO extract that was fractionated by HPLC.

HPLC analysis (Waters, Milford, MA, USA) was conducted with a Vydac C<sub>18</sub> reverse-phase column (Vydac, Hesperia, CA, USA; catalogue # 218TP54) with a flow rate of 1.0 mL min<sup>-1</sup> and a Vydac guard column at wavelength 214 nm (catalogue no. 218GK54, Vydac, Hesperia, CA, USA). As a baseline 18 % ACN (ACN/H<sub>2</sub>O; w/w) was run through the system for at least 10 min prior to sample injection. The sample was loaded and isocratic elution was conducted with 18 % ACN for 45 min to remove all non-polar light fractions. Thereafter, gradient elution was conducted for 90 min with 18 to 82 % ACN. The LCO eluted at 84 to 86 min of HPLC run time. The chemical identity of the LCO was confirmed by FAB-mass spectroscopy and MALDI-TOF mass spectroscopy.

The collected LCO samples were lyophilized (Novalyph Freeze Drying system, model SNL216V, Savant Inc., Holbrook, USA) and stored at -20 °C until further use (Souleimanov et al. 2002a).

### 3.3.3 Production of thuricin 17

*Bacillus thuringiensis* NEB17 was cultured in King's medium B (Proteose peptone no. 3 20 g, K<sub>2</sub>HPO<sub>4</sub> 0.66 g, MgSO<sub>4</sub> 0.09 g, glycerol 0.06 mL, distilled water 1 L) (Atlas 1995). The initial broth inoculum was taken from plated material and grown in 250 mL flasks, containing 50 mL of the King's B medium. The bacterium was cultured at 28 ± 2 °C on an orbital shaker for 48 h, at 150 rpm. A subculture sample of 5 mL was added to 2 L of broth and cultures were grown in 4 L flasks under the same conditions as the initial culture. Bacterial populations were determined spectrophotometrically using an Ultrospec 4050 Pro UV/Visible Spectrophotometer (LKB, Cambridge, UK) at 600 nm (Dashti et al. 1997) 96 h after culture initiation. A cell-free supernatant (CFS), containing the BtNEB17 compound was prepared by centrifuging the bacterial culture at 13,000 g for 10 min on a Sorvall Biofuge Pico (Mandel Scientific, Guelph, ON, Canada). The supernatant was collected and the bacterial compound was detected via analytical-HPLC on a Vydac C:18 reverse phase column (0.46 × 25 cm; 5 µM L<sup>-1</sup>) (catalogue no. 218TP54). The HPLC was fitted with Waters 1525 Binary HPLC pump and a Waters 2487 Dual λ Absorbance detector set (Waters Corporation, Milford, MA, USA) at 214 nm.

For partial purification of the bacterial compound, 2 L of bacterial culture was phase partitioned against 0.8 L butanol for 12 h. The upper butanol layer was collected by rotary evaporation at 50

°C under vacuum. After evaporation, the resulting light brown viscose extract was resuspended in 25 mL of 18% ACN. Prior to HPLC analysis, samples were centrifuged on a Sorvall Biofuge Pico (Mandel Scientific) at 13,000 g for 13 min, and the supernatant was collected for chromatography. HPLC analysis (Waters Corporation) was conducted on a Vydac C:18 reversed-phase column as described above. Conditions of the fractionation chromatography were as follows: 45 min at 18 % ACN, 45–110 min gradient elution with 18–60.4 % ACN, 110–115 min at 60.7–100 % ACN, and 115–120 min at 100–18 % ACN. The HPLC elutions were collected at 1-min intervals (Bai et al. 2002). Preparative HPLC samples were separated into 120-min fractions and were analysed for peaks with retention times between 80 and 82 min as this is when the peptide elutes. The peptide elutes in ~60 % ACN. This material is denoted partially purified bacterial peptide and samples were lyophilized and stored at -20 °C.

### **3.3.4 Germination conditions**

Every experiment described below was repeated twice and the data were pooled for analysis. To disinfest the seed surface before treatment, seeds were soaked in 20 % bleach (6 % sodium hypochlorite, NaOCl) and agitated and rinsed with distilled water. We conducted our experiment in three Phases: in Phase 1, we established which treatment and concentration enhanced *B. napus* cv. Polo germination; in Phase 2, we tested the effect of the  $10^{-6}$  M LCO solution on *B. napus* cvs. 04C204, Polo, and Topas, under optimal and low temperature conditions; in Phase 3, we tested a set of cultivars, under low, medium, and high temperature conditions. For Phase 1 of the experiment, growth cabinets were set to the Association of Official Seed Analysts (1993) standard for *B. napus* germination: 16 h at 20 °C, 8 h at 30 °C, 95% humidity, and zero illumination. For Phase 2, growth cabinets were set 70% humidity, zero illumination, and 24 h at 10 °C, which is a recommended seed bed temperature for spring seeding in Canada (Alberta Agriculture and Development 2012). At 10 °C we previously saw effects of LCO treatment on the germination rate of *Arabidopsis* (Subramanian et al. 2010). For Phase 2, growth cabinets were also set to the ideal germination temperature (25 °C) (Kucera et al. 2005). For Phase 3, growth cabinets were set to low (10 °C), mid-range (20 °C), and high (30 °C), 70% humidity, and zero illumination (Omid et al. 2010). Under a laminar flow hood, using flame-sterilized tweezers, twenty-five seeds were placed into each Petri dish. For Phases 1 and 2, four Petri dishes replicated each treatment, and 3 were used to replicate each treatment applied in Phase 3. Treatment solutions were prepared under sterile conditions, using polyestersulfone (PES)

filtration (Jung et al. 2008). The threshold concentration for the plant perception of microbial peptides and lipo-oligosaccharides is approximately picomolar (Dusenbery 1992). Therefore, for Phase 1 of this experiment, Petri dishes of 25 seeds each were treated with 10 mL of  $10^{-5}$ ,  $10^{-6}$ , or  $10^{-9}$  M LCO solution;  $10^{-9}$ ,  $10^{-10}$ ,  $10^{-11}$ , or  $10^{-12}$  M thuricin 17 solution;  $10^{-5}$ ,  $10^{-6}$ , or  $10^{-9}$  M chitopentaose (Seikagaku Kogyo Co. Ltd., Tokyo) solution; or distilled water. As such, Phase 1 was structured following a partially crossed design with repeated measures in time (Schielzeth and Nakagawa 2013). For Phases 2 and 3, Petri dishes were treated with 10 mL  $10^{-6}$  M LCO solution, or  $10^{-6}$  M chitopentaose solution, or distilled water. Phase 3 included 10 mL  $10^{-11}$  M thuricin 17 solution as a treatment (Prithiviraj et al. 2003). Phases 2 and 3 were factorial design experiments, with an elicitor treatment with 3 levels in Phase 2 and 4 levels in Phase 3, a temperature treatment with 2 levels in Phase 2 and 3 levels in Phase 3, and a cultivar treatment with 3 levels in Phase 2 and 6 levels in Phase 3, and repeated measures in time. After installation into growth cabinets, germination was assessed every 6 h.

### 3.3.5 Statistical analysis

Germination data were analyzed with PROC GLIMMIX in SAS 9.2 (SAS Institute Inc., Cary, NC, USA) with the binomial distribution option and a power covariance structure for thermal time, *i.e.*, growing degree days. Growing degree days were calculated based on this equation:

$$GDD = \left[ \frac{(T_{\max} + T_{\min})}{2} \right] - T_{base} \quad T_{base} = 2$$

SAS code from Piegorsch and Bailer (2005) was used to estimate a form of the Gompertz equation:

$$E = b_0 \times e^{[-e^{[-b_1 - b_2 \times GDD]}]}$$

where  $GDD$  = growing degree days;  $E$  = cumulative germination;  $b_0$  = asymptotic maximum of  $E$  taken as the maximum cumulative germination;  $b_1$  = empirical Gompertz shape parameter; and  $b_2$  = the slope of the curve, the Gompertz time constant. The differences between Gompertz parameters were assessed based on 95% confidence intervals computed by SAS PROC NLIN. The treatment and concentration effects were assessed with Type III Tests of Fixed Effects and differences between the levels were assessed with  $t$ -tests with the LSMEANS statement with

Bonferroni or Scheffé's adjustments for multiple comparisons, respectively.

$$E_i = \frac{b_0}{e}$$

is the germination accumulated by thermal time  $GDD_i$ , at the Gompertz inflection point (Piegorisch and Bailer 2005). SAS PROC UNIVARIATE was used to assess the normality of the distribution of the Gompertz derived indices. We used SAS PROC MIXED to make comparisons when the normality condition was satisfied. When the Gompertz derived indices did not conform to the conventional statistical assumptions, nonparametric Kruskal-Wallis tests, with adjustments for multiple comparisons, were used, *i.e.*, Dunn's tests (Elliot and Hynan 2010).

Only differences at the  $p \leq 0.05$  level were considered significant. SAS PROC UNIVARIATE was used to generate  $n$  values, means ( $\mu$ ), standard errors (se). When biologically interesting differences occur at  $p < 0.1$  these are reported and discussed. Lower and upper limits of Confidence Intervals are indicated by LL and UL, respectively.

### 3.4 Results

The LCO solution at a  $10^{-6}$  M concentration increased the early germination of our conventional canola cultivar, Polo, under standard germination conditions (Fig. 3.1). After Scheffé's adjustment for multiple comparison, the  $10^{-6}$  M level increased the number of germinated seeds, overall, compared to the control and the  $10^{-9}$  M level,  $p = 0.0656$  and  $0.0450$ , respectively. The Gompertz parameters for seed treated with LCO were different from the control ( $p < 0.05$ ) (Table 3.2). The concentration of the treatments had an effect ( $p = 0.0436$ ) on the maximum cumulative germination ( $b_0$ ), but after Scheffé's adjustment for multiple comparison, the superiority of the  $10^{-6}$  M over the  $10^{-9}$  M concentration was only significant at the  $p < 0.1$  level ( $p = 0.0951$ ). The concentration of the treatments also had an effect on the shape parameter ( $b_1$ ) ( $p = 0.0177$ ).

Polo seed treated with a  $10^{-6}$  M concentration of LCO germinated earlier than the controls, as indicated by the lower value of the slope parameter of its germination curve (Gompertz time constant,  $b_2$ ) (Table 3.2). The concentration of the treatments also had an effect ( $p = 0.0029$ ) on

the slope: the  $10^{-5}$ ,  $10^{-6}$ , and  $10^{-11}$  M levels initiated germination earlier than the control, which resulted in lower slopes ( $p = 0.0813$ ,  $0.0144$ , and  $0.0809$  respectively). The  $10^{-9}$  M concentration treatments initiated germination later, but germinated more uniformly to a final level closer to 100 % germination, compared to the  $10^{-5}$  and  $10^{-6}$  M levels, and therefore the slopes of the germination curves differed ( $p = 0.0519$  and  $0.0064$ , respectively). In the 5 to 15 GDD early germination period, 75 % more LCO-treated Polo seed germinated, of 25 seeds per Petri plate, compared to the water-treated controls ( $n_{\text{obs}} = 64$ ;  $\mu_{\mu\text{M LCO}} = 4.9$  seeds,  $se = 2.1$ ;  $\mu_{\mu\text{M chito}} = 2.8$  seeds,  $se = 1.1$ ;  $\mu_{\text{H}_2\text{O}} = 0.8$  seeds,  $se = 0.4$ ; unadjusted  $p_{\text{H}_2\text{O}-\mu\text{MLCO}} = 0.0007$ ).

*T*-tests with Scheffé's adjustment indicated that, at 24 h 10 °C, sister cultivars 02C3 and 02C6 had lower accumulated germination at Gompertz function inflection points ( $E_i$ ) than the other cultivars ( $p < 0.1$ ) and 04C111 had a higher  $E_i$  than 04C204, Polo, and Topas ( $p < 0.05$ ). Under 24 h 10 °C temperature conditions, 02C3 had more ungerminated and dead seed, indicated by lower  $E_i$  values, than 04C111, 04C204, Polo, and Topas ( $p < 0.0001$ ,  $p = 0.0061$ ,  $p < 0.0001$ , and  $p = 0.0008$ , respectively); 02C6 had lower  $E_i$  values than 04C111, 04C204, Polo, and Topas ( $p < 0.0001$ ,  $p = 0.0627$ ,  $p = 0.0004$ , and  $p = 0.0090$ , respectively); and 04C111 had higher  $E_i$  values than 04C204, Polo, and Topas ( $p = 0.0003$ ,  $p = 0.0443$ , and  $p = 0.0022$ , respectively) (Fig. 3.2 and Table 3.3). Consistently, under 24 h 10 °C temperature conditions, 02C3 had fewer seeds germinated than 04C111, 04C204, Polo, and Topas ( $p < 0.005$ ) and 02C6 counts were lower than 04C111 and Polo ( $p < 0.0001$ ). Topas performed relatively well at 24 h 10 °C, compared to its performance under higher temperature conditions (Table 3.4).

*T*-tests with Scheffé's adjustment indicated that cultivar 04C111 had more radicle emergence and survival, indicated by its higher  $E_i$  value, than 02C3 and 02C6 under 24 h 20 °C temperature conditions, and Polo had a higher  $E_i$  than 02C3 under 24 h 20 °C temperature conditions (Dunn's test,  $\alpha = 0.05$ ) (Table 3.3) (Fig. 3.2). Higher  $E_i$  values for 04C111 and Polo under 24 h 20 °C temperature conditions indicated abundant radicle emergence and survival for these cultivars.

Under 24 h 25 °C temperature conditions, Polo and Topas germinated more speedily than 04C204. This difference in speed can be described by the slope,  $b_2$ . The 95% Confidence Limits for  $b_2$ , for 04C204 germination under 24 h 25 °C conditions ( $b_2 = 0.079$ , LL = 0.065, UL = 0.093)

did not overlap with the 95% Confidence Limits for the  $b_2$  parameters for either Polo ( $b_2 = 0.330$ , LL = 0.232, UL = 0.428) or Topas ( $b_2 = 0.340$ , LL = 0.241, UL = 0.440). Refer to Fig. 3.3 for a graphical representation of 04C204's relatively slow rate of germination.

T-tests with Scheffé's adjustment indicated that, at 24 h 30 °C, 02C3 had a lower  $E_i$  than 04C111, 04C204, Polo, and Topas (Table 3.3) ( $p < 0.0001$ ,  $p = 0.0270$ ,  $p = 0.0013$ , and  $p = 0.0134$ , respectively); 02C6 had a lower  $E_i$  than 04C111, Polo, and Topas ( $p < 0.0001$ ,  $p = 0.0071$ , and  $p = 0.0670$ , respectively); and 04C111 had a much higher  $E_i$ , indicating more abundant radicle emergence and survival, as compared to 04C204, Polo, and Topas ( $p < 0.0001$ ,  $p = 0.0009$ , and  $p < 0.0001$ , respectively) (Fig. 3.2) (Table 3.3).

The temperature-by-signal compound treatment interaction was significant ( $p = 0.0142$ ). Therefore, as a component of the interaction, the signal molecule effect was also significant. More (6.9 %) of the thuricin 17-treated seed were germinated per count than controls, at 24 h 10 °C ( $n = 432$ ;  $\mu_{T17*10C} = 7.7$  seeds,  $se = 0.4$ ;  $\mu_{H2O*10C} = 7.2$  seeds,  $se = 0.3$ ;  $p_{T17-H2O} = 0.0596$ ) (Fig. 3.4). At 24 h 30 °C, more (17.2 %) of the chitopentase-treated seed germinated, overall, than either LCO- or water-treated seed ( $n = 234$ ;  $\mu_{LCO*30C} = 5.8$  seeds,  $se = 0.4$ ;  $\mu_{chito*30C} = 6.8$  seeds,  $se = 0.4$ ;  $\mu_{H2O*30C} = 5.8$  seeds,  $se = 0.3$ ;  $p_{chito-LCO} = 0.0048$  and  $p_{chito-H2O} = 0.0333$ , respectively - Fig. 3.5).

### 3.5 Discussion

The challenges to *B. napus* germination are discussed below; they include light, lack of moisture, salinity, seed characteristics, soil compaction, and temperature conditions (Shykewich 1973; Farhoudi et al. 2012). Continuous white light inhibits *B. napus* germination strongly under low temperature conditions and low water volume; *ergo* the seeds were kept in darkness for these experiments. However, Bazańska and Lewak (1986) reported that increasing the amount of water can almost totally eliminate the light-induced inhibition of germination. Seed oil components can also challenge *B. napus* germination, for example, transgenically modified high-stearate *B. napus* has disadvantageous germination characteristics, relative to untransformed controls (Linder et al. 1998). The negative effect of seed oil modification reported by Linder et al. (1998) may be pertain to 04C204's poor germination performance under 24 h 25 °C conditions, and this is discussed below. Furthermore, *Brassica napus* cvs. that produce large

seeds are predicted to be better competitors as seedlings, and have a higher survival rate before competition than seedlings from small seeds (Geritz et al. 1999; Adamski et al. 2009). Although Timiriaziff (cited by Coffman 1923) reported the germination of brassicaceous seed in a block of ice, the optimum temperatures for *B. napus* are in the range of 10 to 30 °C. Low temperature conditions slow the rate of imbibition, damage *B. napus* embryos by chilling injury, and prevent germination, which indicates scarce radicle emergence and death (Omidi et al. 2010). Other work has shown that the germination response of different cultivars to low temperatures have marked non-linearities (Marshall and Squire 1996). The seed of some *B. napus* cultivars remain viable but do not germinate at low temperatures. There have been links made between poor performance at cool temperatures and the low glucosinolate character; the enzymatic hydrolysis of glucosinolates results in a number of products with the potential to inhibit germination (Acharya et al. 1983; Brown and Morra 1996).

Work done by this lab has shown that LCO enhances the germination of Arabidopsis, beans (*Phaseolus vulgaris* [L.]), beets (*Beta vulgaris* [L.]), cotton (*Gossypium hirsutum* [L.]), *G. max*, *O. sativa*, and *Z. mays* under laboratory, greenhouse, and field conditions (Khan 2003; Prithviraj et al. 2003). With respect to thuricin 17, Lee et al. (2009) found that it can enhance *G. max* and *Z. mays* growth. In keeping with those previous findings, our germination assay showed that 10<sup>-6</sup> M LCO treatment encouraged the early germination of *B. napus* cv. Polo, under standard temperature conditions. Previous work has shown that the effects of thuricin 17 can be greater under conditions of abiotic stress (Smith 2010; Subramanian et al. 2010, 2011). Correspondingly, in these experiments thuricin 17 treatment enhanced germination under stressfully cold conditions.

Subsequent assays demonstrated an interesting cultivar-by-temperature interaction for Topas at 10 °C (Table 3.3). Our LCO treatment seems to enhance Topas germination at 24 h 10 °C, but the effect was not statistically significant for the parameters that we calculated. This isn't the first time Topas has been observed to germinate relatively well under cold temperature conditions. Coventry et al. (1988) reported that Topas embryos often had germination rates between 95 and 100 % when treated for 10 days at 4 °C. In light of this positive cultivar-by-temperature interaction, it is worthy of note that early germination and emergence under cold

conditions are important factors that affect yield of crops grown in northern temperate areas.

Whittington (1973) concluded that maternal genetic factors strongly influenced germination rate in *B. napus*. That is, the differences we observed, where maternal differences were known between the cultivars, were not surprising. However, 04C204's relatively slow and incomplete germination, compared to Polo and Topas (Fig. 3.2), is a clear example of the unintended and problematic effects of breeding for higher seed oil contents, whereas its sister cultivar 04C111, which has more seed oil % d.w., performed relatively well under all conditions tested here. The difference in the response of 02C3 and 02C6 to the respective signal molecules, despite the fact that they were produced by the same crosses of double haploid lines, we may deduce to be the product of genomic change during their generations of open pollination, or epigenetic changes. This is not surprising, given the changes to germination observed in other cultivars with altered seed oil contents (Linder et al. 1998). Similarly, under 24 h 20 °C temperature conditions, 04C111 germinated more vigorously as compared to its open pollinated sister cultivar 04C204. This difference between the sister cultivars was detectable by their  $E_i$  values, that is, their accumulated germination at their respective Gompertz function inflection points, under 24 h 30 °C temperature conditions.

We found that chitopentaose encouraged *B. napus* germination generally, and especially under high temperature conditions (30 °C). Treatment with chitopentaose resulted in early and uniform germination, particularly for cv. 04C111 under mid-range temperature conditions (20 °C). These results agree with those of Tsugita et al. (1993 cited in Ohta et al. 2000 [in Japanese]) who used deacetylated chitin (chitosan) to promote brassicaceous *R. sativus* growth, and Guan et al. (2009) who used it to enhance non-brassicaceous *T. aestivum* and *Z. mays* germination. Similarly, Wang et al. (2010) found that chitosan, among other things, enhanced the growth of *B. chinensis*.

Subramanian (personal communication) observed an increase in total abscisic acid (ABA) content in *Arabidopsis* rosettes, 24 h post-treatment with LCO, and other groups have found increased *B. napus* germination due to ABA treatment, *e.g.*, *Brassica napus* germinated more rapidly under low temperature, saline, and water stressed conditions when pre-hydrated and re-dried, or when primed with ABA (Zheng et al.1998; Gao et al. 2002). The constitutive

expression of *Pisum sativum* ABA-responsive 17 cDNA (*ABR17/PR 10.4*) in *B. napus* enhanced germination under cold as well as saline conditions (Dunfield et al. 2007). Other groups have found that SA triggered defense elements, such as pathogen response protein (PR10) and thylakoid ascorbate peroxidase (tAPX), over expressed in *B. napus* seed enhanced germination in saline media (Srivastava et al. 2004; Wang et al. 2011).

These compounds have potential utility for agronomic application to *B. napus*. The method of using LCO as a germination enhancing seed treatment has already been patented (patent number WO2000004778 A1), however it has been not fully investigated for canola. The 6.9 or 17.2 % increases in germination due to thuricin or chitopentase treatment at 24 h 10 and 30 °C, respectively, must be reproduced in field experiments. As such, there seems to be a potential niche available for the use of thuricin 17 and chitopentase to increase germination rates, thereby hasten crop canopy closure, which could inhibit weed seed germination and result in a reduced requirement for herbicide application and increase yield.

### 3.6 Conclusions

The occurrence of microbe-to-plant signal-based communication was verified in the experimental cultivars, as the signal molecules elicited positive seed germination responses. In the 5 to 15 GDD range of the early germination period, seventy-five % more Polo seeds germinated in  $10^{-6}$  M LCO solution than in distilled water (Fig 3.1). Further experimentation with other cultivars and a range of temperature conditions did not discern any other particular differences, following treatment with LCO, thuricin, or chitopentase, under mid-range (24 h 20 °C) and ideal (24 h 25 °C) growing conditions. However, under 24 h 10 °C, dark conditions, treatment with  $10^{-11}$  M thuricin 17 solution increased the number of seeds that germinated by 6.9 %, for all 6 experimental cultivars. Under 24 h 30 °C, dark conditions treatment with  $10^{-6}$  M chitopentase solution increased the number of seeds that germinated by 17.2 %, for all 6 experimental cultivars. Therefore, thuricin 17 and chitopentase enhanced the germination of the 6 experimental cultivars, but their effects depended on the temperature, and the effect of LCO was specific to the Polo cultivar, and was only discernable under ideal temperature conditions.

### 3.7 Tables

**Table 3.1 The seed oil content of the experimental cultivars ( $\pm 2$  % d.w.)**

<i>Brassica napus</i> cultivar	*Seed oil content (% d.w.)
02C3	50.6
02C6	51.2
04C111	53.8
04C204	51.0
Polo	47.2
Topas	42.2

\* University of Manitoba agronomic data

**Table 3.2 The Gompertz function parameters\* that describe the germination of *Brassica napus* cv. Polo**

Treatment	Parameter	Estimate	95% Confidence Limits
μM LCO	$b_0$	22.5910	21.5780 to 23.6041
	$b_1$	-2.2275	-2.9314 to -1.5236
	$b_2$	0.1927	0.1412 to 0.2442
μM chitopentaose	$b_0$	22.7148	21.6932 to 23.7364
	$b_1$	-3.1808	-4.1934 to -2.1682
	$b_2$	0.2377	0.1694 to 0.3060
Distilled water	$b_0$	22.4722	21.8153 to 23.1292
	$b_1$	-5.8124	-7.4615 to -4.1632
	$b_2$	0.4107	0.3063 to 0.5152

\*estimated using SAS PROC NLIN

**Table 3.3 The mean germination accumulated near the midpoint of germination ( $E_i \pm se$ ) for the experimental cultivars, under controlled temperature conditions in the dark.**

Cultivar	Temperature (°C)		
	10	20	30
02C3	$4.9 \pm 0.2^{EF\text{GH}^*}$	$4.4 \pm 0.2^H$	$3 \pm 0.2^I$
02C6	$5.3 \pm 0.2^E$	$4.8 \pm 0.2^{EFGH}$	$3.3 \pm 0.2^I$
04C111	$8.2 \pm 0.2^A$	$7.6 \pm 0.2^B$	$7 \pm 0.2^{BC}$
04C204	$6.3 \pm 0.2^D$	$5.2 \pm 0.2^{EF}$	$4.4 \pm 0.2^{GH}$
Polo	$7.1 \pm 0.2^{BC}$	$6.3 \pm 0.2^D$	$5 \pm 0.2^{EFG}$
Topas	$6.7 \pm 0.2^{CD}$	$5.2 \pm 0.2^E$	$4.5 \pm 0.2^{FGH}$

\* superscripts indicate least significant difference ( $p < 0.05$ ) (Saxton 1998). Rows and columns were all compared.

**Table 3.4 Differences between cultivar  $\times$  temperature ( $^{\circ}\text{C}$ ) combinations**

$\text{cv}_1 \times \text{temp}_1$	- $\text{cv}_2 \times \text{temp}_2$	Estimate	*Scheffé-adjusted p
04C204 $\times$ 25	- 04C204 $\times$ 10	-0.5235	0.4257
04C204 $\times$ 25	- Polo $\times$ 25	-1.7479	<.0001
04C204 $\times$ 25	- Polo $\times$ 10	-1.6282	<.0001
04C204 $\times$ 25	- Topas $\times$ 25	-1.8515	<.0001
04C204 $\times$ 25	- Topas $\times$ 10	-2.9512	<.0001
04C204 $\times$ 10	- Polo $\times$ 25	-1.2244	<.0001
04C204 $\times$ 10	- Polo $\times$ 10	-1.1047	0.0009
04C204 $\times$ 10	- Topas $\times$ 25	-1.3280	<.0001
04C204 $\times$ 10	- Topas $\times$ 10	-2.4278	<.0001
Polo $\times$ 25	- Polo $\times$ 10	0.1197	0.9984
Polo $\times$ 25	- Topas $\times$ 25	-0.1036	0.9991
Polo $\times$ 25	- Topas $\times$ 10	-1.2033	0.0002
Polo $\times$ 10	- Topas $\times$ 25	-0.2232	0.9705
Polo $\times$ 10	- Topas $\times$ 10	-1.3230	<.0001
Topas $\times$ 25	- Topas $\times$ 10	-1.0998	0.0011

\*estimated by SAS PROC GLIMMIX with a LSMEANS statement that specified ADJUST=SCHEFFE.

### 3.8 Figure captions

**Figure 3.1** Gompertz curves ( $E = b_0 \times e^{[-e^{[-b_1 - b_2 \times GDD]]}]$ ) overlaying a scatterplot showing the numbers of *B. napus* cv. Polo seeds germinated. The 95% Confidence Limits for Gompertz parameters, calculated for seeds treated with a  $10^{-6}$  M concentration of LCO (broken dashed line,  $R^2 = 0.94$ ), did not overlap those of controls (solid line,  $R^2 = 0.97$ ). The germination of seeds treated with a  $10^{-6}$  M concentration of chitopentaose is shown by the dashed line ( $R^2 = 0.94$ ).

**Figure 3.2** Accumulated germination at Gompertz function inflection points ( $E_i$ ). Bars indicate standard errors.

**Figure 3.3** Germination of *B. napus* cultivars under 24 h 25 °C temperature conditions. Replicates were selected daily at random and removed from the growth cabinets for measurement of the radicles (data not shown). Treatments are indicated on the graph as follows: LCO =  $\mu$ M lipo-chitooligosaccharide solution; chito =  $\mu$ M chitopentaose solution; and H<sub>2</sub>O = distilled water control.  $R^2_{04C204,H_2O} = 0.88$ ;  $R^2_{04C204,LCO} = 0.88$ ;  $R^2_{04C204,chito} = 0.79$ ;  $R^2_{Polo,H_2O} = 0.73$ ;  $R^2_{Polo,LCO} = 0.73$ ;  $R^2_{Polo,chito} = 0.76$ ;  $R^2_{Topas,H_2O} = 0.70$ ;  $R^2_{Topas,LCO} = 0.72$ ;  $R^2_{Topas,chito} = 0.75$ .

**Figure 3.4** *Brassica napus* cultivars treated with  $10^{-11}$  M thuricin 17 solution (T17) and grown under 24 h 10 °C temperature conditions compared to controls (H<sub>2</sub>O).  $R^2_{02C3,H_2O} = 0.84$ ;  $R^2_{02C3,T17} = 0.91$ ;  $R^2_{02C6,H_2O} = 0.91$ ;  $R^2_{02C6,T17} = 0.84$ ;  $R^2_{04C111,H_2O} = 0.96$ ;  $R^2_{04C111,T17} = 0.95$ ;  $R^2_{04C204,H_2O} = 0.92$ ;  $R^2_{04C204,T17} = 0.91$ ;  $R^2_{Polo,H_2O} = 0.91$ ;  $R^2_{Polo,T17} = 0.94$ ;  $R^2_{Topas,H_2O} = 0.77$ ;  $R^2_{Topas,T17} = 0.91$ .

**Figure 3.5** The germination of *B. napus* cvs. treated with LCO and chitopentaose, at 24 h 30 °C.  $R^2_{02C3,H_2O} = 0.55$ ;  $R^2_{02C3,LCO} = 0.45$ ;  $R^2_{02C3,chito} = 0.83$ ;  $R^2_{02C6,H_2O} = 0.76$ ;  $R^2_{02C6,LCO} = 0.67$ ;  $R^2_{02C6,chito} = 0.70$ ;  $R^2_{04C111,H_2O} = 0.88$ ;  $R^2_{04C111,LCO} = 0.78$ ;  $R^2_{04C111,chito} = 0.96$ ;  $R^2_{04C204,H_2O} = 0.75$ ;  $R^2_{04C204,LCO} = 0.83$ ;  $R^2_{04C204,chito} = 0.73$ ;  $R^2_{Polo,H_2O} = 0.86$ ;  $R^2_{Polo,LCO} = 0.89$ ;  $R^2_{Polo,chito} = 0.87$ ;  $R^2_{Topas,H_2O} = 0.89$ ;  $R^2_{Topas,LCO} = 0.74$ ;  $R^2_{Topas,chito} = 0.62$ .

### 3.9 Figures

Figure 3.1

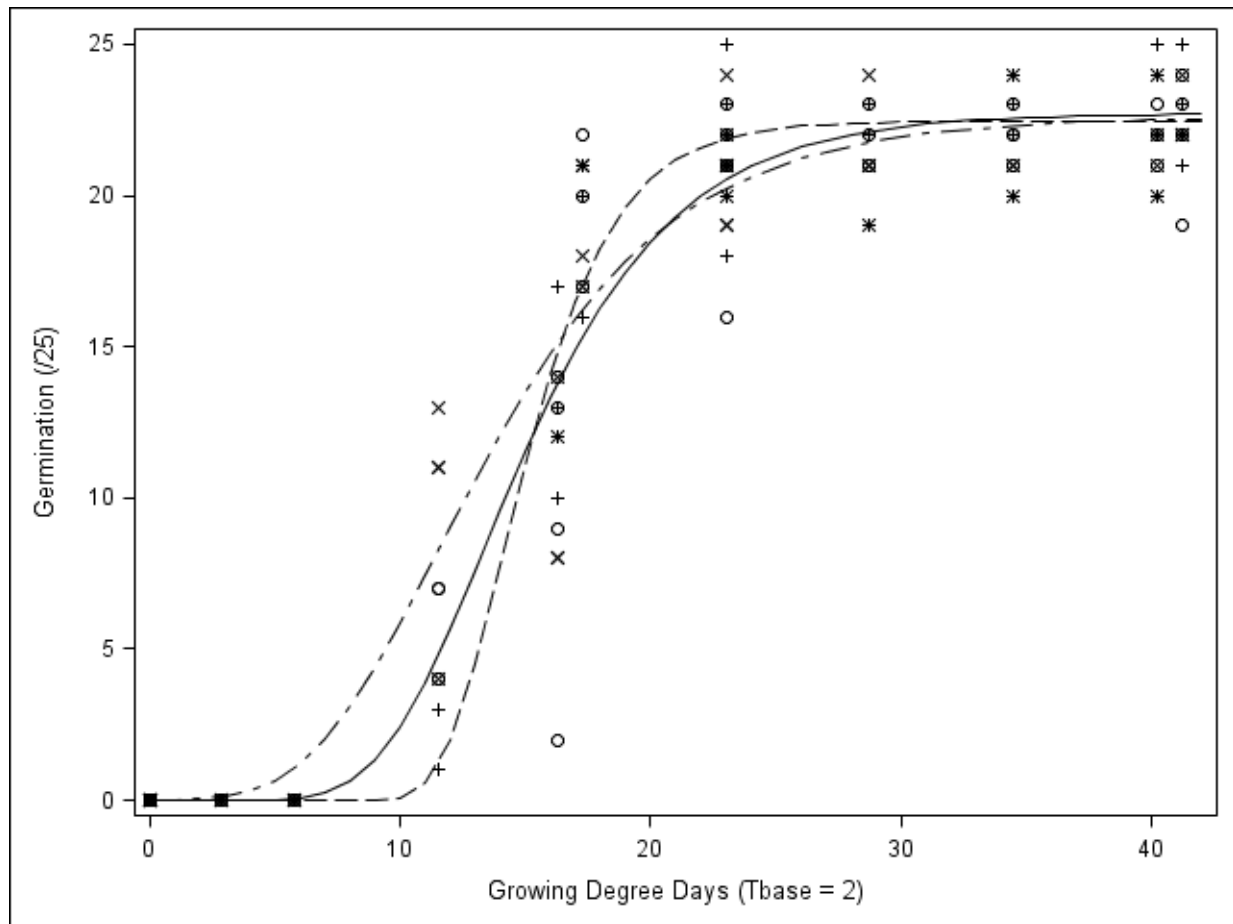


Figure 3.2

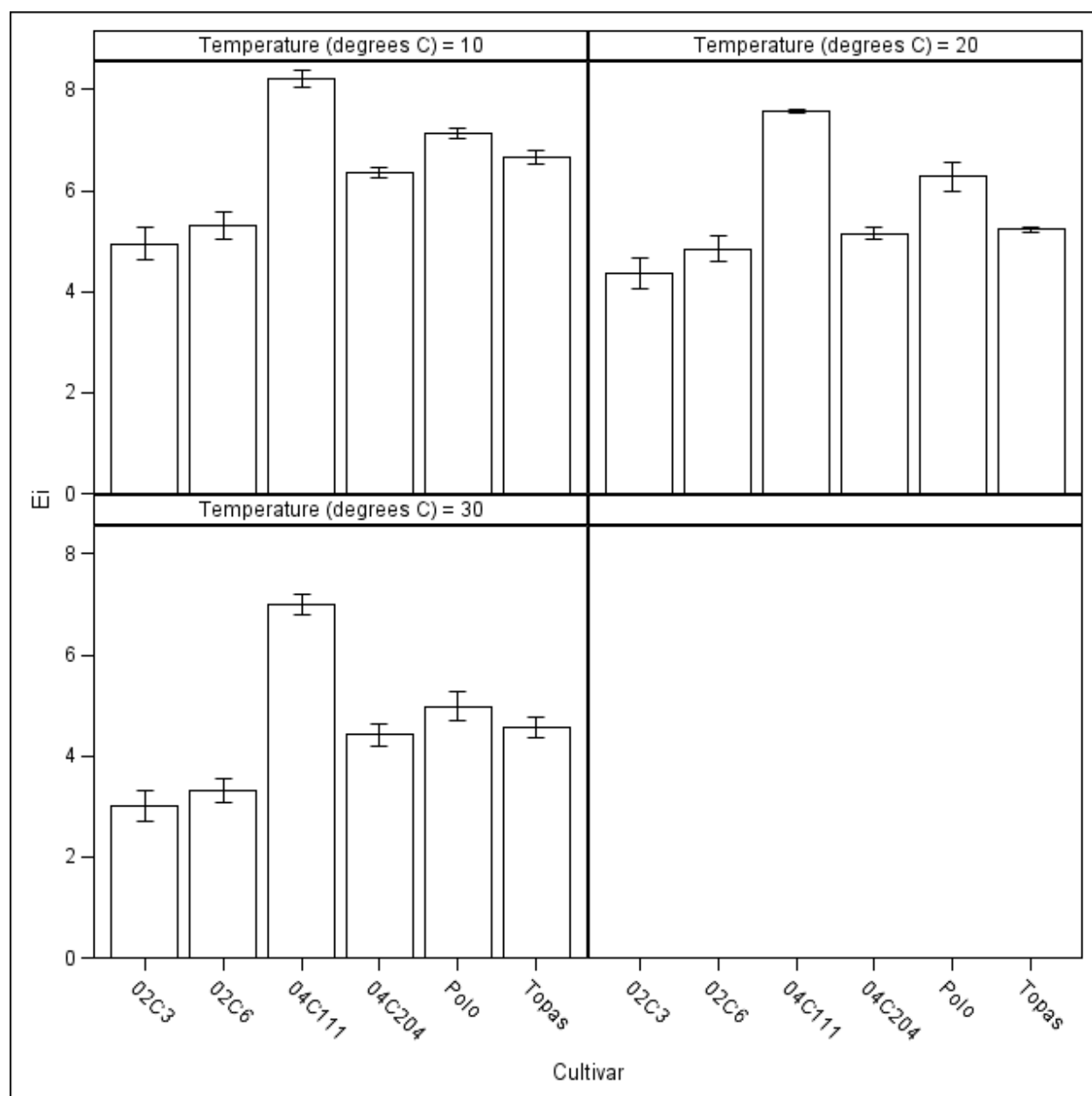


Figure 3.3

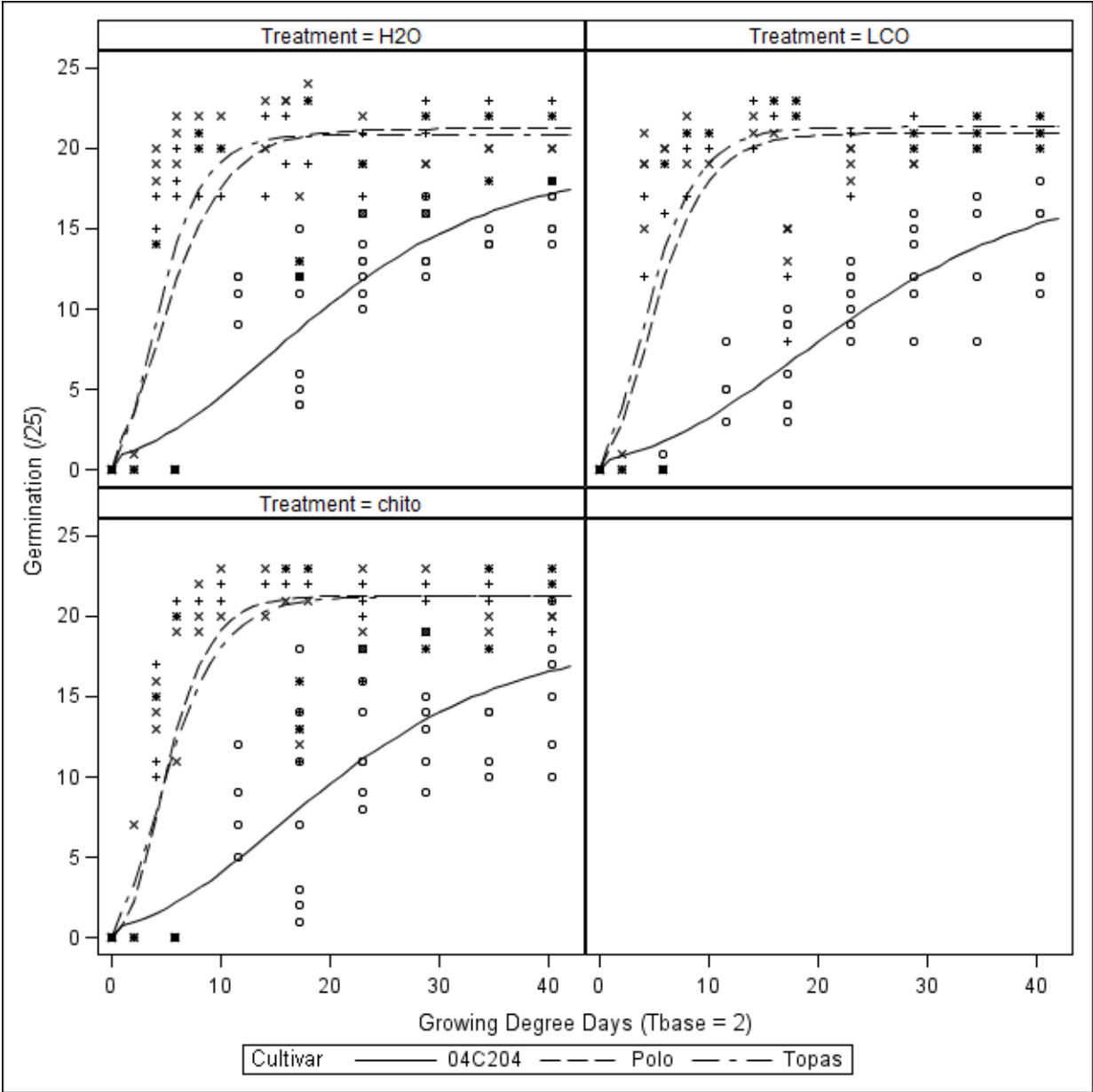


Figure 3.4

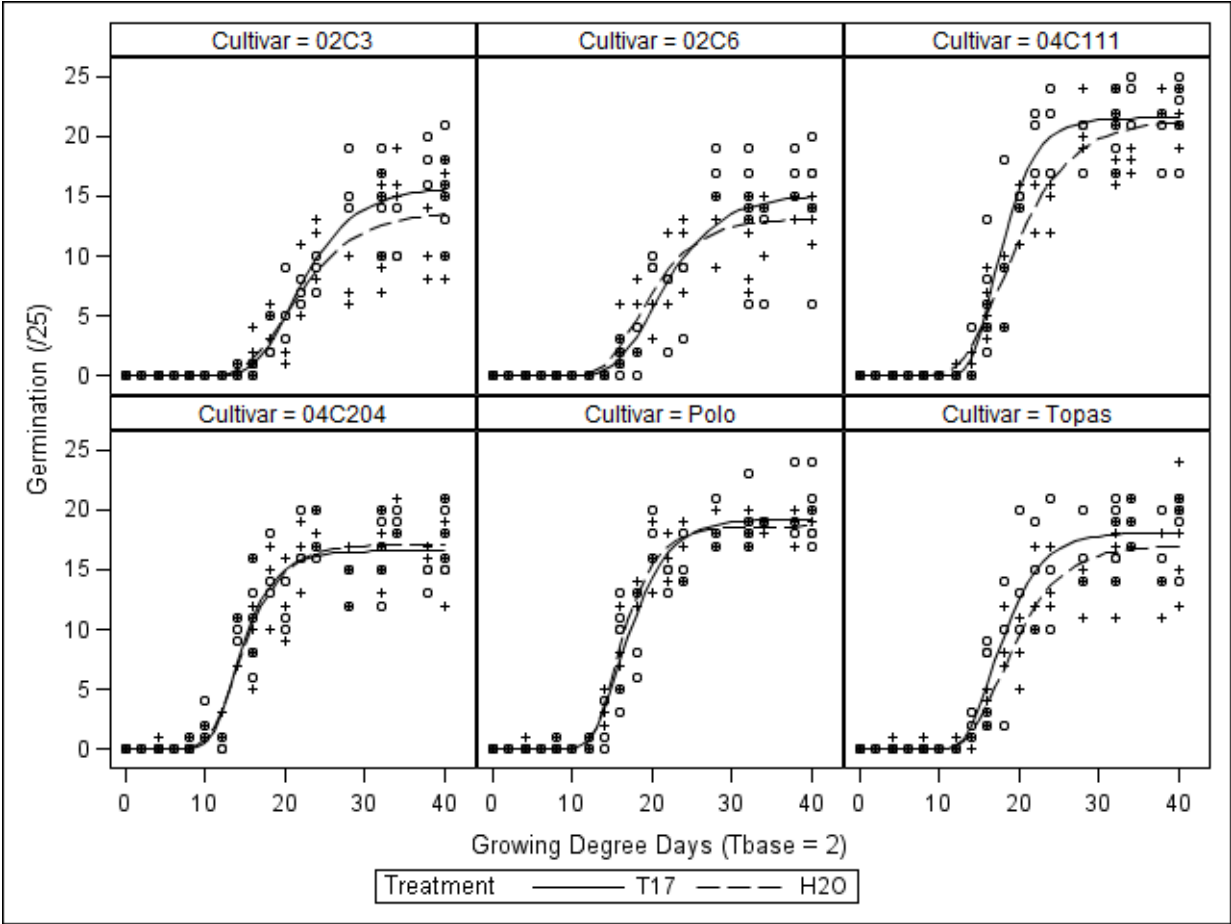
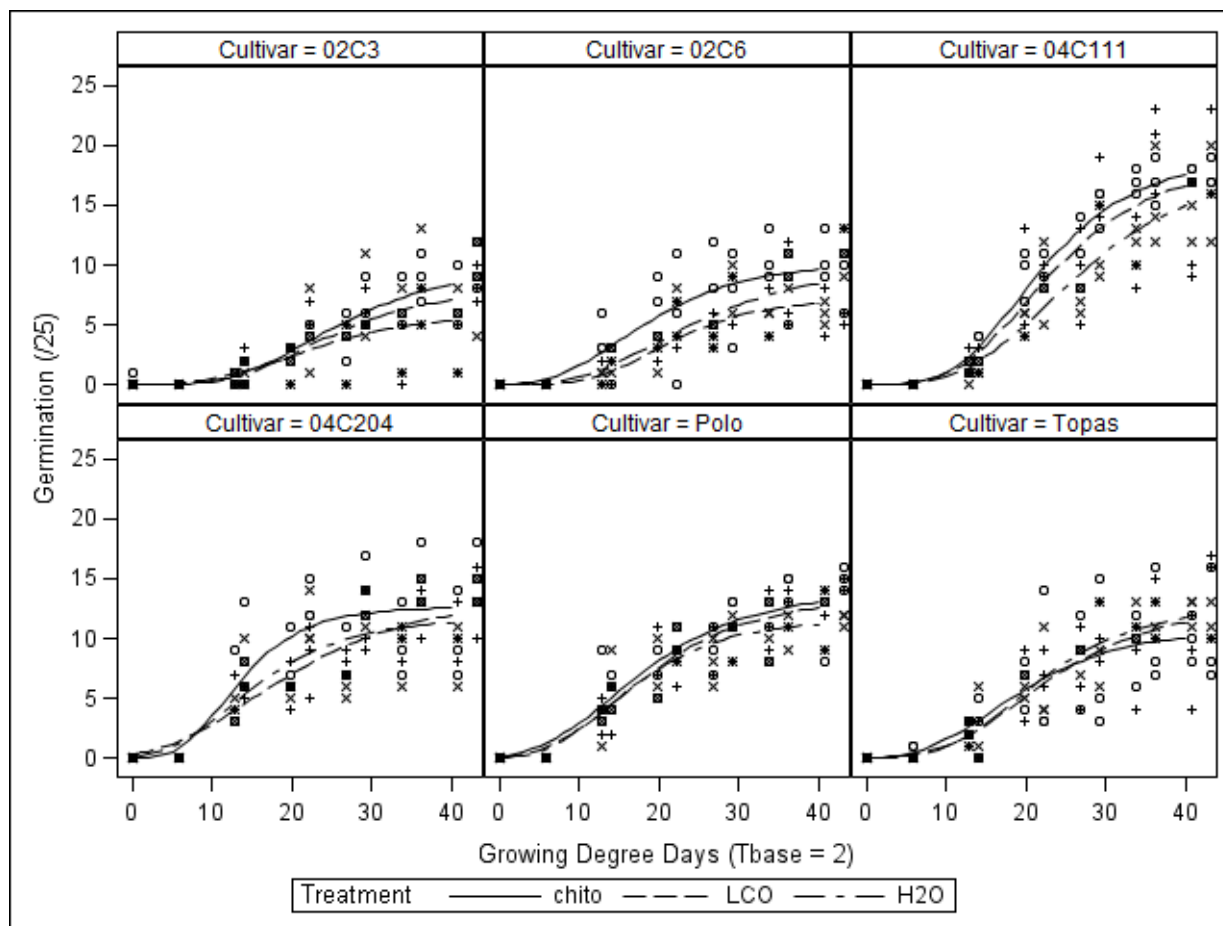


Figure 3.5



#### 4 Preface to Section 4

Section 4 is a manuscript by Schwinghamer, Souleimanov, Dutilleul, and Smith will be submitted to Environmental and Experimental Botany. The literature cited in this section is listed in the reference section (page 160). Each figure caption, figure, and table is presented at the end of the section.

This section addresses a potential role for a set of microbe-to-plant signal compounds as plant growth regulators that enhance seedling emergence and affect the architecture (stem height, primary root length, and petiole lengths) of young canola plants. There is a clear advantage to early emergence and increased coverage of the surface of the growth medium; if these results are reproducible under agricultural conditions, they could allow the crop early soil surface coverage in the field and increase the crop's competitive advantage over weeds. The results of these experiments include: under combined cold ( $10/4^{\circ}\text{C}$ ) and prohibitively saline ( $200 \times 10^{-3} \text{ M NaCl}$ ) conditions, treatment with  $10^{-9} \text{ M}$  thuricin 17 increased the fresh weight of 38-day-old plants grown in plant culture vessels by 58.9 mg ( $p = 0.0013$ ); and under moderately high ( $30/30^{\circ}\text{C}$ ) temperature and saline ( $100 \times 10^{-3} \text{ M NaCl}$ ) conditions, the  $10^{-9} \text{ M}$  thuricin 17 treatment also eliminated instances of secondary embryogenesis among 20-day-old plants, which otherwise was observed to occur at a rate of 1 extra plant from 10 seeds ( $\alpha = 0.05$ ).

The thesis consists of 4 papers. The first focused on the beginning of development, *i.e.*, germination. This is the second, which focuses on subsequent growth and early development. The third focuses on development right through to yield, but using a rapid system; and the fourth focus on development, including plant architecture through to yield with conventional genotypes.

## **Irrigation with lipo-chitooligosaccharide Nod Bj V (C18:1, MeFuc) and thuricin 17 solutions regulates the phyllotaxy, height, and mass of young canola (*Brassica napus* [L.] plants**

### **4.1 Abstract**

Given that canola (*Brassica napus* L.) forms neither arbuscular mycorrhizal (AM) nor nitrogen-fixing symbioses, it has not been much evaluated in its capacity to perceive and respond to microbe-to-plant signals. Previously, the *Bradyrhizobium japonicum* lipo-chitooligosaccharide (LCO) (Nod Bj V [C18:1, MeFuc]) and *Bacillus thuringiensis* bacteriocin thuricin 17 were shown to enhance the germination and growth of other crop species. Two experimental systems, a peat pellet system and alternatively large plant culture vessels, were used to grow *B. napus* plants in controlled environment chambers. The effects of irrigation with  $10^{-6}$  M LCO solution and  $10^{-11}$  M thuricin 17 solution on biofuel *B. napus* cv. 04C111 and conventional *B. napus* cv. Topas architecture and biomass were assessed using the a peat pellet system. From a range of concentrations and temperatures, optimal points of interaction were identified by a Central Rotatable Composite Designed experiment for the enhancement of Topas fresh weight, dry weight, and plant developmental stage. The large plant culture vessels were used to assess the effect of the continuous presence of thuricin 17 and LCO treatment on 04C111 plant growth, under a range of salinity conditions. In the peat pellet system, when irrigated with  $10^{-6}$  M LCO solution and grown at constant moderately high (30/30 °C) temperature conditions, the young plants produced  $1 \pm 0.2$  ( $\alpha = 0.05$ ) more leaf than water- or signal-treated plants grown at ideal (25/20 °C) temperature conditions. Treatment with  $10^{-11}$  M thuricin 17 reduced the heights of 20-day-old 04C111 plants grown in plant culture vessels, similar in effect to salt stress conditions ( $100 \times 10^{-3}$  M NaCl). However, our  $10^{-11}$  M thuricin 17 treatment increased the fresh weight of 38-day-old 04C111 plants grown in plant culture vessels under combined salt ( $200 \times 10^{-3}$  M NaCl) and cold (10/4 °C) stress conditions by  $58.9 \pm 17.7$  mg ( $p_{\text{diff}} = 0.0013$ ). Secondary embryogenesis, where a second plant either grew out of the hypocotyl of the first plant, or was produced by twinning within the seed, occurred at a rate of once per 10 seeds (10 %). The continuous presence of  $10^{-9}$  M thuricin 17 treatment eliminated secondary embryogenesis among plants grown in  $100 \times 10^{-3}$  M NaCl media.

## 4.2 Introduction

Rhizobia-legume and rhizobia-*Parasponia* symbiotic interactions are mediated by LCO signal molecules, which require the activity of three specific enzymes, encoded by the *nodABC* genes that are present in all characterized rhizobia (reviewed by Oldroyd et al. 2010; Op den Camp et al. 2011). *nodABC* genes, which synthesize the *N*-acetylglucosamine (GlcNAc) oligomer backbone of the LCO molecule, can be found on the chromosome of *Bradyrhizobium japonicum*, whereas, for most rhizobia, they are often be found in symbiotic plasmids or islands that may be mobilized by lateral transfer among rhizobia (Sullivan and Ronson 1998; Rogel et al. 2001). For most legumes, lipo-chitooligosaccharides play a pivotal role for host-symbiont specificity and the induction of the early steps of infection and root nodule organogenesis, including symbiotic gene activation leading to mitotic reactivation of the cortical cells, and the formation of pre-infection threads (Oldroyd et al. 2010). The arbuscular mycorrhizal (AM) fungus *Glomus intrradices* also secretes sulphated and nonsulphated simple LCOs that stimulate the formation of AM symbioses with plants in the *Fabaceae*, *Asteraceae*, and *Umbelliferae* families (Maillet et al. 2011). Lipo-chitooligosaccharides that trigger root nodule organogenesis are called Nod factors (Dénarié and Cullimore 1993). The Dg1 symbiont of Durango root (*Datisca glomerata* [C. Presl] Baill.) contains *nodABC*-like genes, which suggests that it too uses Nod factor-like compounds during the infection process (Persson et al. 2011). Nod Bj V (C18:1, MeFuc) (MW 1415) is a Nod factor that is produced abundantly by *Bradyrhizobium japonicum*. It is a fatty acylated chitin pentasaccharide, *i.e.*, five GlcNAc residues with a 2-*O*-methyl-L-fucosyl residue attached to the 6 position of the reducing end GlcNAc. In aqueous solution, it forms large molecular weight aggregates, when the concentration is greater than millimolar, but it is likely monomeric at physiological concentrations (Groves et al. 2005). The lipid decoration of the LCO molecule is involved in the specificity of perception (Gough and Cullimore 2011). The relative orientation of the oligosaccharide and acyl moieties can range from being extended to essentially perpendicular to being quasi-parallel (Groves et al. 2005; Morando et al. 2011).

Brassicaceous plants have neither rhizobial symbiotic nor AM associations, and are thought not to recognize the other bioactive molecules that are important for the establishment of a functional AM symbiosis (Navazio et al. 2007; Genre et al. 2013). However, plants that do not host rhizobia may nevertheless perceive LCO. Recently published work indicated that a smaller brassicaceous plant, *Arabidopsis*, responded to LCO treatment by reducing levels of

FLAGELLIN-SENSING 2 (FLS2) receptors located on the plasma membrane, and suppressing its microbial-associated molecular pattern (MAMP) -triggered reactive oxygen species (ROS) production (Liang et al. 2013). Furthermore, microbial-associated molecular patterns, such as LCO, may also enter plants via epidermal fissures generated by the emergence of lateral or adventitious roots, such as are typical of doubled haploid line *B. napus* varieties, as was reported by Sprent (2008) for the infection of tropical aquatic legumes of the *Aeschynomene* genus by *Bradyrhizobium* spp. Lipo-chitooligosaccharide has been observed to be accumulated, at relatively low levels of uptake, and concentrated and immobilized by binding sites in the cell wall of root hairs, which become saturated by micromolar concentrations, before interaction with any receptors, such as lysin motif (LysM)-containing chitin elicitor receptor kinases (RLKs) in the plasma membrane (Goedhart et al. 2000). Lysin motif RLKs are critical for the perception of Nod factors and the legume–rhizobial symbiotic interaction (Madsen et al. 2003; Radutoiu et al. 2003). Five LysM RLK genes were identified in brassicaceous *Arabidopsis thaliana* [L.] Heynh, including RLK1/chitin elicitor receptor kinase 1 (CERK1), which induces plant defense after binding to penta- to octameric chitin, which it does more strongly than to shorter chitin oligomers (Petutschnig et al. 2010).

Previous work has shown that Nod Bj V (C18:1, MeFuc) from *B. japonicum* USDA110 can stimulate cell division of wild soybean (*Glycine soja* P1468397 [Siebold and Zucc.]) and siratro (*Macroptilium atropurpureum* [DC.] Urb.) at  $10^{-10}$  M (Sanjuan et al. 1992). Nod Bj V (C18:1, MeFuc) plays a role in seed germination and growth in diverse plants (Souleimanov et al. 2002b; Khan et al. 2008). Lipo-chitooligosaccharides have also been shown to have mitogenic and morphogenic effects similar to cytokinins and inhibitors of auxin transport (Schmidt et al. 1993; Dénarié et al. 1996).

Recent ultra-performance liquid chromatography – electron spray ionization quantification showed that LCO treatment can increase plant total abscisic acid (10.19 %) and salicylic acid (15.00 %), and decrease indolylacetic acid (-49.68 %), cytokinin (-36.24 %), gibberellic acid (-19.41 %), and jasmonic acid (-33.66 %) content in *Arabidopsis* rosettes 24 h post treatment (Subramanian, personal communication). Natural plant growth regulators (PGRs) like salicylic acid (SA) and abscisic acid (ABA) can influence the plant response to abiotic stress (Meng et al. 2009; Ullah 2012). Ullah et al. (2012) found that the foliar application of  $10^{-5}$  M SA can

increase the accumulation of the plant stress marker proline, as well as increase leaf soluble proteins, and protect chlorophyll *a* and carotenoids in cvs. Rainbow and Dunkeld. Correspondingly, Kazemi et al. (2010) found that treatment with SA increased the above ground dry weight of *B. napus* cv. PF plants (Kazemi et al. 2010). On the other hand, high levels of ABA are correlated with high photosystem II excitation pressure and cold acclimation during development and germination (Rapacz et al. 2003).

Thuricin 17 is produced by *Bacillus thuringiensis* NEB17. The sequence of the peptide was published by Lee et al. (2009). Although thuricin 17 has not been confirmed as a signal in the phloem, peptides such as thuricin 17 could generate or amplify messages, and act as signals themselves within the phloem of *B. napus* (Giavalisco et al. 2006). Thuricin 17 is a class IId bacteriocin with a broad range of antimicrobial activity against related bacterial species (Gray et al. 2006a). Class IId bacteriocins produced by *Bacillus* strains are highly effective inducers of the plant defense-related enzymes phenylalanine ammonia lyase, guaiacol peroxidase, ascorbate peroxidase, superoxide dismutase, and polyphenol oxidase (Jung et al. 2011). Previous work has shown that thuricin 17 can enhance the growth of soybean (*Glycine max* [L.] Merr) and corn (*Zea mays* [L.]) (Lee et al. 2009).

Owing to the very short growing season in Canada, particularly in the prairie regions, *Brassica napus* is usually seeded in the early spring months, when the temperature is below optimum, so that the plants are able to reach maturity without experiencing reduced yield due to the occurrence of frost during the plant's seed filling stage of development. The production of *B. napus* is further imperiled by its low emergence rate, that is, only 50 % of planted *B. napus* seed typically emerge under agricultural conditions (Harker et al. 2003). The salinization of agricultural land is an increasing challenge as cultivation expands and encroaches upon previously marginal lands. Sodium chloride (NaCl) is the most ubiquitous cause of soil salinity, and concentrations of NaCl have been observed to inhibit *B. napus* seed germination incrementally (Shahbazi et al. 2011). Reduced germination rates can increase the susceptibility of seedlings to soil borne pathogens, *e.g.*, phoma stem canker (blackleg) (*Leptosphaeria maculans*), decrease the vigor of young plants, delay maturity, and delay the depletion of red light by the crop canopy, which would otherwise inhibit weed seed germination (Silvertown 1980; Médiène et al. 2011). Reduced

seedling emergence and slow ground-cover, reduces the competitive advantage of the crop over weeds.

Higher plant biomass is an indication of a higher net assimilation rate and can favour higher crop yields (Taiz and Zeiger 1998). Adverse temperature conditions, salinity, and water logging reduce the biomass, growth, and yield of *Brassica* species (Bandehagh et al. 2011, Tunçtürk et al. 2011). And, although *Brassica napus* is sensitive to saline conditions, it produces greater fresh and dry biomass under saline growing conditions, compared to other brassicaceous crops (Ashraf and McNeilly 1990).

In northern temperate areas, the vigorous growth and early maturity of young crop plants are important factors that affect yield. In spring canola cultivars, plant height has been positively associated with seed yield, and among early-maturing winter cultivars in South China, plant height was significantly correlated with the height of the first lateral branch, the length of the main raceme, and the numbers of siliquae on the main raceme, all of which are factors that contribute to yield (Aytac et al. 2008; Lu et al. 2011). Shorter plants can produce increased vegetative growth and yield, owing to increased tolerance to lodging under unfavorable conditions (Olenjniczak and Adamaska 1999).

Plant leafiness is a key factor for the interception of radiation and carbon assimilation, and is positively correlated to seed yield (Clarke and Simpson 1978; Denoroy et al. 1998). Rapid early light interception and crop canopy closure improves early biomass accumulation on an area basis, provides shade that can inhibit weed growth in an agricultural environment, and thereby improves plant growth and crop yield (Silvertown 1980; Gunasekera et al. 2006). Leaf removal and shading of *B. napus* plants at the reproductive stage reduced yield, owing to the strong relationship of leaf area with siliquae numbers and seeds per siliquae, but Shen et al. (2005) indicated that assimilate translocation is the most critical limiting factor for seed yield in *B. napus*, rather than source and sink organs (Tayo and Morgan 1979; Wang et al. 2011). In any case, more leaves on plants would increase the supply of photosynthetic assimilate, which controls the growth of competing sinks and the relationship between source and sink.

Whereas the balance of ABA and GA and the concentration of ABA in imbibed seeds are factors that determine germination, and endogenous and external signals can influence hormone levels,

and whereas previous studies established the tolerance of *B. napus* to saline water irrigation (Yanagawa and Fujimaki 2013), the objectives were to assess in a peat pellet growing system, whether or not the emergence of plants will be increased due to elicitor (LCO and thuricin 17) irrigation treatment, as compared to water-treated controls at (day/night) 10/4, 25/20, 30/30 °C conditions; and to determine plant height, leaf number, and petiole lengths, after treatment with the elicitors of interest, as compared to water-treated controls at 10/4 (cold temperature stress) , 25/20 (ideal), and 30/30 °C (warm temperature stress) conditions. The objective of the central rotatable composite design (CRCD) experiment was to determine the optimal points for the interaction of LCO concentration with temperature by finding ridges of maximum response. The objective of the plant culture vessel system experiments was to quantify embryogenesis, final fresh and dry mass, plant height, petiole extension, and leaf number of plants treated with thuricin 17 in saline growing media.

## **4.3 Methods**

### **4.3.1 Plant material**

For the germination assay, open pollinated high erucic acid *B. napus* cv. 04C111 was used; this genotype was produced using conventional crosses and pedigree (Dr. P. McVetty, University of Manitoba). The crosses that produced 04C111 are presented in standard breeder's notation, where '/' represents one cross, and increasing numbers of '/' marks indicates previous crosses, here: HiQ////Apollo///86LL141//Tatyoan/R83-14. *Brassica napus* cv. Topas (SWAB, Svalöv, Sweden) was also used. It is a commercial-quality cultivar.

### **4.3.2 Peat pellets assay**

This experiment was repeated twice and the data were pooled for analysis. Lipochitooligosaccharide and thuricin 17 were produced as described in section 3.3.2 and 3.3.3. The first experiment followed a 2-factor factorial design. Peat pellets (Jiffy Products, Shippagan, NB) were soaked until saturated in their respective treatment solutions of either  $\mu\text{M}$  LCO,  $10^{-11}$  M thuricin 17, or distilled water control (48 pellets per 3 L solution). Eight pellets were situated in each  $22.9 \times 15.2 \times 5.1$  cm tray (Rubbermaid, Malaysia) and 3 trays were allocated to each treatment per experimental replicate in time. As such, in each replication of the experiment, there were approximately 24 plants per treatment-by-temperature group. Trays were installed

into controlled environment cabinets set to 25/20, 10/4, and 30/30 °C, 14 h day / 10 h night. A single 04C111 seed was planted into each peat pellet. Trays were randomized immediately and re-randomized every 2 days thereafter. Plants were grown for 11 days and irrigated with either  $10^{-6}$  M LCO solution, or  $10^{-11}$  M thuricin 17 solution, or distilled water. Trays were irrigated uniformly, each with 300 mL of their respective treatment solution, in each controlled environment cabinet as needed. Fresh and dry weights of plants were obtained using a Mettler AE-166 laboratory scale. Plant architectural metrics (stem height, primary root length, and petiole lengths) were measured using an electronic caliper with digital display (Mastercraft, Canada). All data presented here were produced by experimentation repeated in time.

A second experiment was set out as a 2-factor CRCD, using *B. napus* cv. Topas seeds planted individually into peat pellets. To make the regression coefficients orthogonal to one another, and to give the experimental design the property of being rotatable, the plants were irrigated with  $10^{-12}$ ,  $10^{-11.12}$ ,  $10^{-9}$ ,  $10^{-6.88}$ ,  $10^{-6}$  M concentrations of LCO solution. The plants grew for 2 weeks under a controlled 14 h day / 10 h night diurnal cycle, under a range of controlled temperature conditions (4, 8, 17, 26, and  $30 \pm 2$  °C). Therefore the factorial combinations were equally spaced around the circumference of a circle. Coordinates that have the same radius have the same standard error (Box and Hunter 1957). To produce the 4 and 8 °C conditions, it was necessary to eliminate and reduce the illumination, respectively. However, the germination of canola is not inhibited by low light or darkness (Bazańska and Lewak 1986). Final measurements were made with the instruments described above.

#### **4.3.3 Cold temperature plant culture vessels assay**

This experiment was repeated twice and the data were pooled for analysis. Thuricin 17 was produced as described in section 3.3.3. The third experiment followed a 2-factor factorial design. Half strength MSB5 was prepared with 0.8 % low-melt agarose, 1 % sucrose, and pH adjusted with KOH and HCl to 5.8. Eight flasks of saline MSB5 media (0, 100, 200, or  $400 \times 10^{-3}$  M NaCl) were autoclaved and  $10^{-9}$  M concentrations of thuricin 17 were added, using a PES filter, under sterile conditions at a laminar flow bench. Thuricin 17 was not added to controls. Treatments were poured into scrubbed and autoclaved reusable plant culture vessels (Magenta® GA 7, U.S. Pat. 4,358,908) that are used usually to grow plantlets from 'germinated' microspore derived embryos to a relatively large size. There were 4 vessel replicates per treatment. The

media was left under the hood to solidify overnight. 04C111 seeds were surface sterilized in 1.5 mL microcentrifuge tube (Fisher Scientific, USA) using 20 % bleach (6 % sodium hypochlorite, NaOCl). Seeds were agitated using a Vortex Mixer (Fisher Scientific, USA) and rinsed with autoclave-sterilized distilled water until odourless. Using autoclaved tweezers, three seeds were planted into each vessel. While planting, the tweezers were intermittently flame sterilized and sterilized with 70 % ethanol. The plant culture vessels were installed into controlled environment cabinets and randomized. Controlled environment cabinets provided a diurnal cycle of 14 h illumination at 10 °C and 10 h darkness at 4 °C. Plant culture vessels were re-randomized every 2 days. Plants were grown for 38 days, at the end of this time plant growth, development, and architectural metrics were assessed with the instruments described above. Plants were dried in coin envelopes at 60 °C for 3 days and weighed.

#### **4.3.4 High temperature plant culture vessels assay**

This experiment was repeated four times, twice with each of agar and low-melt agarose media. The data were pooled for analysis. Thuricin 17 was produced as described in section 3.3.3. The fourth experiment followed a 2-factor factorial design. *Brassica napus* cv. 04C111 plants were grown in both agar and low-melt agarose MSB5 media in scrubbed and autoclaved plant culture vessels. Controlled environment cabinets were set to 30 °C and a diurnal cycle of 14 h illumination and 10 h darkness. Plants were grown for 20 days in lowmelt agarose-based MSB5 media that was prepared with and without  $10^{-9}$  or  $10^{-11}$  M thuricin 17 concentrations, and with a range of salinities (0, 0.05, 50, 100, or  $200 \times 10^{-3}$  M NaCl). After fresh weight and architectural metrics (stem height, primary root length, and the lengths of the plant's petioles) were gathered with the instruments described above, and the plants were dried in coin envelopes at 60 °C for 3 days and weighed.

#### **4.3.5 Statistical analysis**

Principal component analysis (PCA) of the ranked variables was produced using PROC RANK in SAS 9.3 and SAS PROC PRINCOMP (SAS Institute Inc., Cary, NC, USA). The accompanying figures were produced using Canoco 5 (Biometris, Wageningen University and Research Centre, The Netherlands). When plant architectural metrics, and fresh or dry weights, conformed to conventional statistical assumptions, SAS PROC MIXED was used to produce *t*-tests with p-values to assess the significance of differences between treatment least square

means, with Bonferroni's or Scheffé's adjustments for multiple comparisons where appropriate. In cases where a chi-squared test indicated nonhomogeneous variance or the Shapiro-Wilk statistic indicated that the distribution of the residuals was not normal, an outlier was identified by its high internally studentized residual and removed based on its high leverage statistic or Cook's distance. In some cases, the plant data per vessel or per tray was pooled and retested. The Box Cox method was used to identify the appropriate power transformation, but these were not sufficient to normalize the data. Nonparametric Kruskal-Wallis tests were applied with adjustments for multiple comparisons where necessary, *i.e.*, Dunn's test. The CRCDD dataset was analyzed using SAS code from Bowley (2008). Values for the number of observations are indicated by "n." Values for the mean ( $\mu$ ) and standard error (se) are reported. Results where  $p < 0.1$  are reported. In all cases, the p-values or coefficient of determination ( $\alpha$ ) values are indicated. Correlation coefficients are indicated using  $\rho$ .

## 4.4 Results

### 4.4.1 Peat pellet assay

More LCO-treated seed emerged ( $n = 51$ ,  $\mu_{\text{LCO}} = 90.2\%$ ,  $se = 0.0$ ), as compared to thuricin 17-treated seed ( $n = 49$ ,  $\mu_{\text{T17}} = 69.4\%$ ,  $se = 0.1$ ,  $\alpha = 0.05$ ) or controls ( $n = 50$ ,  $\mu_{\text{H}_2\text{O}} = 72.0\%$ ,  $se = 0.1$ ,  $\alpha = 0.1$ ), under 10/4 °C temperature conditions. However, at 10/4 °C, plants that were irrigated with thuricin 17 grew to be slightly taller than LCO-treated plants, after 11 days of growth ( $n_{\text{LCO}} = 51$ ,  $\mu_{\text{LCO}} = 11.397$  mm,  $se = 0.821$ ;  $n_{\text{T17}} = 49$ ,  $\mu_{\text{T17}} = 12.321$  mm,  $se = 0.968$ ;  $p_{\text{LCO-T17}} = 0.0880$ ).

Plants irrigated with a  $10^{-6}$  M LCO solution that were grown under 30/30 °C conditions were multifoliate, in that they produced  $1 \pm 0.2$  ( $\alpha = 0.05$ ) more leaf than water- or signal-treated plants grown at ideal (25/20 °C) temperature conditions, but they did not produce significantly more leaves than the other plants that were grown at 30/30 °C. Although irrigation with either LCO or thuricin 17 solutions at 25/20 °C produced plants with as many leaves as controls or thuricin 17-treated plants grown under 30/30 °C, those plants did not produce as many leaves as LCO-treated plants at 30/30 °C ( $\alpha = 0.05$ ) (Table 4.1). Therefore, at 30/30 °C, the thuricin 17-treated plants and the controls produced more leaves than the controls grown at 25/20 °C, but not more than the signal-treated plants ( $\alpha = 0.05$ ) (Table 4.1). *Brassica napus* cv. 04C111 plants

grown under 10/4 °C temperature conditions for 11 days did not produce leaves, other than the cotyledons.

The comparisons of petiole lengths between treatment groups within temperature levels revealed that there were no significant effects on petiole lengths after 11 days of growth (Table 4.1; Fig. 4.1).

The results of the PCA from the peat pellet assay are shown in figures 4.2 and 4.3, and the correlations are shown in Table 4.2. The PCA indicated that, in this data set, for the multiple traits measured on experimental groups differing in the presence and absence of the LCO and thuricin 17 treatments, variability was associated with multiple sources. The first six principal components displayed eigenvalues greater than 1, and according to Kaiser's rule were not trivial. The first PC accounted for 35.91 % of the total variance, the second PC accounted for 10.57 % of the total variance, and the third PC accounted for 8.69 % of the total variance. Although the first PC did not show loading  $> 0.5$  for any of the variables, it was positively correlated to the temperature ( $\rho = 0.68987$ ,  $p < 0.0001$ ), above ground fresh weight ( $\rho = 0.91578$ ,  $p < 0.0001$ ), above ground dry weight ( $\rho = 0.93769$ ,  $p < 0.0001$ ), primary stem height ( $\rho = 0.55778$ ,  $p < 0.0001$ ), leaf number ( $\rho = 0.94471$ ,  $p < 0.0001$ ), and plant developmental stage ( $\rho = 0.94299$ ,  $p < 0.0001$ ). The first PC was also positively correlated to the lengths of the petioles: the longest ( $\rho = 0.93195$ ,  $p < 0.0001$ ), the second-longest ( $\rho = 0.92714$ ,  $p < 0.0001$ ), the third-longest ( $\rho = 0.71222$ ,  $p < 0.0001$ ), the fourth-longest ( $\rho = 0.36958$ ,  $p < 0.0001$ ), and the fifth-longest ( $\rho = 0.11511$ ,  $p = 0.0254$ ). Similarly, the second PC did not show loading  $> 0.5$  for any of the variables, but it was positively correlated to the presence of thuricin 17 ( $\rho = 0.23167$ ,  $p < 0.0001$ ), the presence of LCO ( $\rho = 0.45683$ ,  $p < 0.0001$ ) and negatively correlated with the control treatment ( $\rho = -0.69265$ ,  $p < 0.0001$ ). The second PC was also positively correlated with the number of plants per tray ( $\rho = 0.42529$ ,  $p < 0.0001$ ), the height of the secondary stem ( $\rho = 0.19750$ ,  $p < 0.0001$ ), the number of cotyledons ( $\rho = 0.35705$ ,  $p < 0.0001$ ) the presence of adventitious roots ( $\rho = 0.22161$ ,  $p < 0.0001$ ), and the lengths of the third-longest petiole ( $\rho = 0.15411$ ,  $p < 0.0027$ ), the fourth-longest petiole ( $\rho = 0.29673$ ,  $p < 0.0001$ ) and the fifth-longest petiole ( $\rho = 0.12548$ ,  $p < 0.0148$ ). The second PC was also negatively correlated with the above ground fresh weight ( $\rho = -0.32541$ ,  $p < 0.001$ ), the above ground dry weight ( $\rho = -0.30870$ ,  $p < 0.0001$ ), the primary stem height ( $\rho = -0.19859$ ,  $p < 0.0001$ ), the plant developmental stage ( $\rho = -$

0.13796,  $p < 0.0001$ ), leaf number ( $\rho = -0.14378$ ,  $p < 0.0052$ ), and the lengths of the longest petiole ( $\rho = -0.34316$ ,  $p < 0.0001$ ), the second-longest petiole, ( $\rho = -0.28881$ ,  $p < 0.0001$ ), the third-longest petiole ( $\rho = 0.15411$ ,  $p = 0.0027$ ), the fourth-longest petiole ( $\rho = 0.29673$ ,  $p < 0.0001$ ), and the fifth-longest petiole ( $\rho = 0.12548$ ,  $p = 0.0148$ ). The third principle component showed high positive loading for the LCO treatment and negative loading for the thuricin 17 treatment (Figs. 4.2 and 4.3). The presence of an adventitious root, typically a marker for plant juvenility and sensitivity to auxin (*e.g.*, De Klerk 1996), was positively and significantly correlated with the presence of LCO in the irrigation solution ( $\rho = 0.13243$ ,  $p = 0.0053$ ) (Table 4.2), indicating that LCO treatment was, among the many other expected factors, playing a meaningful role in the development of these plants. This tells us that, while LCO treatment is known to reduce the total plant content of auxin, it can trigger the development of tissues that are usually characteristic of increased sensitivity to auxin.

#### 4.4.2 Central rotatable composite design experiment

The quadratic of LCO concentration had a significant effect on above-ground fresh weights. Ridges of maximum response indicated that to obtain higher above-ground fresh weights, near-nanomolar concentrations of LCO ( $10^{-8.81}$  M) would provide the greatest effect at moderately high levels of temperature (30 °C) (Fig. 4.4). The quadratic of LCO concentration affected the dry weights of 2-week-old Topas plants. Ridges of maximum response indicated that to obtain heavier dry weights, near-nanomolar concentrations of LCO ( $10^{-8.83}$  M) would provide the best effect at moderately high levels of temperature (30 °C) (Fig. 4.5).

The quadratic of LCO concentration had affected plant developmental stage achieved by Topas plants after 2 weeks of growth (Sylvester-Bradley 1985). A ridge of maximum response indicated that, to obtain advanced plant developmental stages, after 2 weeks of growth,  $10^{-12}$  M concentrations of LCO would be effective at mid-range levels of temperature (17 °C) (Fig. 4.6).

#### 4.4.3 Cold temperature assay

Nonparametric tests indicated that the thuricin 17 treatment increased the fresh weight of plants 58.9 mg, 38 DAP compared to controls under stressfully low (10/4 °C) temperature conditions and prohibitively stressful salinity ( $200 \times 10^{-3}$  M NaCl) ( $n_{T17} = 22$ ;  $\mu_{T17} = 171.7$  mg,  $se = 17.7$ ;  $n_0 = 24$ ,  $\mu_0 = 112.8$  mg,  $se = 9.3$ ;  $p_{0-T17} = 0.0013$ ). Considering  $\alpha = 0.1$ , nonparametric Kruskal-

Wallis tests also showed that the thuricin 17-treated plants were slightly (0.277 mm) taller after 38 d of growth in  $200 \times 10^{-3}$  M NaCl media ( $n_{T17} = 23$ ;  $\mu_{T17} = 6.367$  mm,  $se = 0.349$ ;  $n_0 = 24$ ,  $\mu_0 = 6.090$  mm,  $se = 0.244$ ;  $p = 0.0528$ ).

Under the doubly stressful low temperature (10/4 °C) and saline ( $100 \times 10^{-3}$  M NaCl) conditions, thuricin 17 treatment slowed plant development, *i.e.*, plants that were grown for 38 days in low-melt agarose media with a  $10^{-9}$  M concentration of thuricin 17 had approximately 1 less leaf, and were therefore relatively oligophyllous, compared to controls ( $n_{T17} = 24$ ;  $\mu_{T17} = 1.3$  leaves,  $se = 0.2$ ;  $n_0 = 24$ ,  $\mu_0 = 2.0$  leaves,  $se = 0.1$ ;  $p_{0-T17} = 0.0180$ ), and the second-longest petioles produced by thuricin 17-treated plants grown under  $100 \times 10^{-3}$  M NaCl conditions were slightly (0.655 mm) shorter, and when  $\alpha = 0.1$  is considered, relatively sessile ( $n_{T17} = 24$ ;  $\mu_{T17} = 1.042$  mm,  $se = 0.219$ ;  $n_0 = 24$ ,  $\mu_0 = 1.697$  mm,  $se = 0.177$ ;  $p = 0.0655$ ).

The results from the PCA of our measured variables, from the 38-day-old plants grown under 10/4 °C temperature conditions indicated that, in this data set, for the multiple traits measured on experimental groups differing in the presence and absence of thuricin 17 treatments, variability was associated with multiple sources. The first five PCs displayed eigenvalues greater than 1. Therefore, Kaiser's rule retained the first five PCs as they were not trivial. The first PC accounted for 43.63 % of the total variance, the second PC accounted for 19.65 % of the total variance, the third PC accounted for 8.00 % of the total variance, and the fourth PC accounted for 5.74 % of the total variance. The first PC was strongly negatively correlated to the concentration of NaCl ( $\rho = -0.93549$ ,  $p < 0.0001$ ) and strongly positively correlated to the number of leaves ( $\rho = 0.93294$ ,  $p < 0.0001$ ), fresh weight ( $\rho = 0.84884$ ,  $p < 0.0001$ ), dry weight ( $\rho = 0.86937$ ,  $p < 0.0001$ ), the primary root length ( $\rho = 0.95008$ ,  $p < 0.0001$ ), the length of the longest petiole ( $\rho = 0.92933$ ,  $p < 0.001$ ), the length of the second-longest petiole ( $\rho = 0.91213$ ,  $p < 0.0001$ ), and the length of the third-longest petiole ( $\rho = 0.73524$ ,  $p < 0.0001$ ). The first PC was also positively correlated to the number of cotyledons ( $\rho = 0.56709$ ,  $p < 0.0001$ ) and the number of plants ( $\rho = 0.52760$ ,  $p < 0.0001$ ). The second PC was strongly positively correlated to the concentration of NaCl ( $\rho = 0.83912$ ,  $p < 0.0001$ ), and strongly negatively correlated to the number of leaves ( $\rho = -0.75578$ ,  $p < 0.0001$ ), fresh weight ( $\rho = -0.76140$ ,  $p < 0.0001$ ), dry weight ( $\rho = -0.78708$ ,  $p < 0.0001$ ), primary root length ( $\rho = -0.86260$ ,  $p < 0.0001$ ), the length of the longest petiole ( $\rho = -0.80033$ ,  $p < 0.0001$ ), and the length of the second-longest petiole ( $\rho = -$

0.76512,  $p < 0.0001$ ). The second PC was also negatively correlated to the length of the third-longest petiole ( $\rho = -0.50630$ ,  $p < 0.0001$ ). The third PC had positive loading for the number of cotyledons, and it was subsequently labeled the “polycot” component (Table 4.3). The fourth and fifth PCs had strong positive loadings for the concentration of thuricin 17, and they were labeled “T17A” and “T17B” (Table 4.3). Thus, the polycotyledonous characteristic was a relatively major source of variability in the growth and development of the young plants. Added to this are clearly separate effects due to the concentration of thuricin 17.

#### 4.4.4 High temperature assay

There were growth and developmental alterations to thuricin 17-treated 04C111 plants that were grown for 20 days under 30/30 °C temperature conditions. The results from high temperature experiments were produced using multiple datasets from repeated experiments that used both agar and low-melt agarose media.

Under  $100 \times 10^{-3}$  M NaCl conditions, the  $10^{-9}$  M thuricin 17 treatment regulated the occurrences of polyembryogeny or secondary embryogenesis, where a second plant either grew out of the hypocotyl of the first plant, or was produced by twinning within the seed. Seeds grown in vessels of  $10^{-11}$  M thuricin 17 or control saline media produced more than 1 plant seed<sup>-1</sup> ( $n_{nM} = 27$ ,  $\mu_{nM} = 1.0$  plant seed<sup>-1</sup>,  $se_{nM} = 0.0$ ;  $n_{pM} = 13$ ,  $\mu_{pM} = 1.1$  plant seed<sup>-1</sup>,  $se_{pM} = 0.0$ ;  $n_0 = 35$ ,  $\mu_0 = 1.1$  plant seed<sup>-1</sup>,  $se_0 = 0.0$ ;  $\alpha = 0.05$ ). That is, the  $10^{-9}$  M thuricin 17 treatment eliminated secondary embryogenesis, which otherwise occurred at a rate of 1 extra plant from 10 seeds (10%).

After 20 days of growth, nonparametric comparisons across the salinity levels revealed that the  $10^{-9}$  M thuricin-treated plants grouped with the controls and plants grown under  $0.05 \times 10^{-3}$  M NaCl conditions, which grew taller than the plants grown under  $100 \times 10^{-3}$  M NaCl conditions, but the plants treated with  $10^{-11}$  M thuricin 17 were not different in height from any treatment group at any salinity level (Table 4.4, Fig. 4.7). Similarly, comparisons between the signal concentration levels within salinity level groups did not reveal any differences in the numbers of leaves produced after 20 days of growth. However, there were significant differences due to particular factor combinations (Table 4.4, Fig. 4.8). There was no difference due to the treatment concentration on the total of petiole lengths that was discernible within each salinity level. However, when grown with  $10^{-9}$  M thuricin 17, plants grown in nonsaline media and  $0.05 \times 10^{-3}$

M NaCl media produced longer sums of petiole lengths plant<sup>-1</sup> than the controls and 10<sup>-9</sup> M thuricin 17-treated plants grown under 100 × 10<sup>-3</sup> M NaCl conditions (Table 4.4, Figs. 4.9 and 4.10).

The PCA of the measured variables from the plants grown under 30/30 °C temperature conditions was used to extract the components. The first five components displayed eigenvalues greater than 1. Therefore, according to Kaiser's rule, the first five PCs were not trivial. The first PC accounted for 35.92 % of the total variance, the second accounted for 13.75 % of the total variance, the third accounted for 10.32 %, the fourth accounted for 7.99 %, and the fifth accounted for 7.06 % of the total variance. The first PC was strongly positively correlated to the number of leaves ( $\rho = 0.93274$ ,  $p < 0.0001$ ), the length of the longest petiole ( $\rho = 0.92746$ ,  $p < 0.0001$ ), the length of the second-longest petiole ( $\rho = 0.93946$ ,  $p < 0.0001$ ), the length of the third-longest petiole ( $\rho = 0.90800$ ,  $p < 0.0001$ ), and the length of the fourth-longest petiole ( $\rho = 0.80703$ ,  $p < 0.0001$ ). The first PC was also positively correlated to the height of the primary stem ( $\rho = 0.52575$ ,  $p < 0.0001$ ) and the length of the fifth-longest petiole ( $\rho = 0.56969$ ,  $p < 0.0001$ ). The second PC had positive loadings for the lengths of the seventh-longest and eighth-longest petioles, it was therefore labeled the “radial symmetry” component (Table 4.5). The radial symmetry component was negatively correlated to the number of leaves ( $\rho = -0.52955$ ,  $p < 0.0001$ ), the length of the longest petiole ( $\rho = -0.68270$ ,  $p < 0.0001$ ), the length of the second-longest petiole ( $\rho = -0.69923$ ,  $p < 0.0001$ ), and the length of the third-longest petiole ( $\rho = -0.58322$ ,  $p < 0.0001$ ). The third PC had positive loadings for the numbers of stems and the numbers of cotyledons. The third PC was subsequently labeled the “secondary embryogenesis” PC. The plant characteristics of secondary vascular structure and altered phyllotaxy can indicate high levels of auxin or sensitivity to auxin. The fourth PC had high positive loading for sodium chloride concentration, and it was therefore labeled “NaCl”. The fifth PC had high positive loading for the concentration of thuricin 17, and it was labeled “T17”. Therefore, the major sources of variability were components having to do with individual plant growth. The saline growth conditions and thuricin 17 treatment contributed variability separately.

## 4.5 Discussion

Stressfully high temperature conditions can impair plant development and result in fewer organs such as leaves (Stone 2001; Barnabás et al. 2008). Previous work has shown that the effects of

LCO and thuricin 17 can be greater under conditions of abiotic stress (Smith 2010; Subramanian et al. 2010, 2011). Although the hormonal changes triggered by LCO in *Arabidopsis* rosettes indicated a response that was like Systemic Acquired Resistance, no hypersensitive response elicited by the treatment was observed among the young plants. The LCO treatment triggered adventitious root development, which is a marker for plant juvenility (De Klerk 1996). Correspondingly, the suppressed-immune response of *Arabidopsis* to LCO was characterized recently by Liang et al. (2013), including reduced levels of FLAGELLIN-SENSING 2 (FLS2) receptors located on the plasma membrane, and suppressed MAMP-triggered ROS production. In the experimental results reported here, the  $10^{-6}$  M LCO irrigation treatment interacted with moderately high (30/30 °C) temperature conditions to produce approximately 1 more leaf, 11 DAP. Therefore, the high temperature  $\times$  LCO treatment interaction was considered to produce multifoliate plants as compared to the 25/20 °C water-treated controls. The Sylvester-Bradley (1985) stages of plant development were also advanced by 0.1 rank for those plants. The experiment with Topas indicated the optimum point to increase leaf number for this cultivar will be at a lower temperature (17 °C) and  $10^{-12}$  M LCO concentration. Approximately  $10^{-12}$  M concentrations are at the threshold for the plant perception of microbial peptides and lipooligosaccharides (Dusenbery 1992).

Bilgili et al. (2011) found the numbers of leaves produced by brassicaceous species was inversely related to the salt concentration of the growing media. However, the slightly saline ( $0.05 \times 10^{-3}$  M NaCl) treatment seems to have been within the optimum range for *B. napus* growth. Plants grown in vessels under slightly saline and moderately high (30/30 °C) temperature conditions produced more leaves than salt ( $100 \times 10^{-3}$  M NaCl) stressed plants, when treated with either  $10^{-9}$  or  $10^{-11}$  M thuricin 17 (Table 4.4; Fig. 4.9). As such, both of the treatment molecules have the potential to increase plant leafiness, and thereby increase the plant's interception of radiation and carbon assimilation. However, when grown in peat under moderately high (30/30 °C) temperature conditions, and under low (10/4 °C) temperature conditions in plant culture vessels, the  $10^{-11}$  M thuricin 17 treatment inhibited leaf growth, and promoted oligophylly, compared to controls.

The petiole length determines the position of the leaf within the canopy. Under 25/20 °C temperature conditions, the  $10^{-6}$  M LCO irrigation treatment increased the lengths of the longest

petioles of 04C111 plants, to such a degree that they were statistically indistinguishable from the petioles of thuricin 17-treated plants grown under 30/30 °C temperature conditions (Table 4.1). Therefore, the LCO-treated plants were considered to be relatively petiolate at 25/20 °C. Kurepin et al. (2007) subjected similarly young *B. napus* plants to low red to far red ratio treatment, and produced longer, etiolated petioles, as expected for plants subjected to shade. The promotion of a form that is relatively multifoliate and petiolate is anticipated to be competitive in the agricultural environment. At the critical stage of early plant growth, young plants with more leaves and longer petioles will cover a relatively larger area of the growth surface and dominate neighbouring weeds. Fast and early crop canopy closure can provide weed-inhibiting light conditions at the soil surface to reduce herbicide inputs, reduce selection pressure for weed resistance, improve yield, improve quality, and increase agricultural *B. napus* profitability (Górski 1975; Silvertown 1980).

Shahbazi et al. (2011) found salt treatments caused significant reductions in shoot fresh weight, except at the  $50 \times 10^{-3}$  M level. Other groups have shown that, under  $150 \times 10^{-3}$  M NaCl conditions, *Brassica napus* plants survive but show poor tolerance and marked symptomatic effects such as reduced weight of green parts and inhibited to growth (Biligili et al. 2011; Tunçtürk et al. 2011). Indeed, Yanagaw and Fujimaki (2013) reported that biomass production was proportional to the amount of transpiration and the plant's capability to continue to take up water from the soil. Transpiring under stress is the essence of tolerance (Yanagaw and Fujimaki 2013). Therefore, under prohibitively saline ( $200 \times 10^{-3}$  M NaCl) and cool (10/4 °C) conditions, where the thuricin 17 treatment increased the fresh weight of young 04C111 plants by 58.9 mg, it may have done so in part by increasing transpiration and tolerance to salt. The CRCD experiment indicated that, to obtain higher above-ground fresh weights of the Topas cultivar, in the absence of any salt stress, near-nanomolar concentrations of LCO in the irrigation solution would provide the largest positive effects at a moderately high temperature (30 °C).

Typically, plant height is an agronomic selection variable for salinity tolerance (Noble and Rogers 1992). As such, the increased height of  $10^{-9}$  M thuricin 17-treated plants, which were grown in  $200 \times 10^{-3}$  M NaCl media and cold temperature conditions, may be an indication of elevated tolerance to an inclement environment. Other groups have observed the heights of mature *B. napus* plants decrease with increasing salinity, as did the heights of the relatively

immature 04C111 plants (*e.g.*, Ashraf and Naqvi 1992; Shahbazi et al. 2011). Under moderately warm conditions, the  $10^{-11}$  M thuricin 17 treatment shortened, and reduced the instances of secondary embryogenesis of, 20-day-old 04C111 plants to the level of plants grown under saline ( $100 \times 10^{-3}$  M NaCl) conditions. Smaller plants may be less vulnerable to stress because they have less surface area. The reduction of plant height is the target of plant growth retardants, such as PGRs in the triazole family, which are widely applied on *B. napus* under current agricultural production regimes. Many groups, including Olenjniczak and Adamaska (1999) reported that reduction in *B. napus* height cause an increase in vegetative growth and grain yield because of tolerance to lodging under unfavorable conditions. Recently, Beauclair et al. (2009) reduced shoot growth with micromolar 1-aminocyclopropane carboxylic acid (ACC), a precursor of ethylene. However, some groups have attributed high yields of some *B. napus* cultivars to their height, among other characteristics (Tahir et al. 2007; Tohidi Moghadam et al. 2011).

#### 4.6 Conclusions

In conclusion, irrigation solutions of  $10^{-6}$  M LCO and  $10^{-11}$  M thuricin 17 interacted with controlled temperature conditions to affect the growth, architecture, and biomass of 04C111 sown in peat pellets. Under cool (10/4 °C) temperature conditions, plants that were irrigated with LCO solution emerged 18.2 % ( $\alpha = 0.1$ ) more than water-treated controls, but thuricin 17 treatment produced slightly (0.924 mm,  $p < 0.1$ ) taller plants than LCO treatment, in the peat pellet system. Under combined cold (10/4 °C) and prohibitively saline ( $200 \times 10^{-3}$  M NaCl) conditions, treatment with  $10^{-9}$  M thuricin 17 increased the fresh weight of 38-day-old plants grown in plant culture vessels by 58.9 mg ( $p = 0.0013$ ). Under moderately high (30/30 °C) temperature and saline ( $100 \times 10^{-3}$  M NaCl ) conditions, the  $10^{-9}$  M thuricin 17 treatment also eliminated instances of secondary embryogenesis among 20-day-old plants, which otherwise was observed to occur at a rate of 1 extra plant from 10 seeds ( $\alpha = 0.05$ ). The interaction of 30/30 °C temperature conditions with LCO irrigation can produce plants that are multifoliate, in that they produced  $1.0 \pm 0.2$  more leaf than the 25/20 °C water-irrigated controls (Table 4.1), and this was considered to be a competitive form for the newly emerged crop's domination of the field surface at the early stage of growth. Irrigation with  $10^{-11}$  M thuricin 17 reduced the lengths of petioles of 11 day-old 04C111 plants grown under 30/30 °C temperature conditions (Table 4.1). These experiments have identified concentrations of LCO and thuricin 17 that show promise as plant growth regulators and irrigation treatments for use in specific agricultural and climatic contexts

to promote the successful establishment of the crop at the early stages of growth. Future experiments should assess whether or not these changes to plant growth due to LCO or thuricin 17 treatments can enhance the production of seed and seed oil by these *B. napus* cultivars.

## 4.7 Tables

**Table 4.1 Leaf number and lengths of petioles produced by 04C111 plants grown in a peat pellet system, in controlled environment cabinets, 11 DAP**

Temperature (day/night °C)	Irrigation treatment	Number of leaves		Longest petiole length		Second-longest petiole length		Third-longest petiole length (mm ± se)	
		<i>n</i>	(count ± se)	<i>n</i>	(mm ± se)	<i>n</i>	(mm ± se)	<i>n</i>	(mm ± se)
25/20	10 <sup>-6</sup> M LCO	99	2.0±0.1 <sup>bc*</sup>	59	13.558±0.582 <sup>bc</sup>	49	8.149±0.464 <sup>b</sup>	11	4.129±0.708 <sup>b</sup>
	10 <sup>-11</sup> M Thuricin 17	99	2.0±0.1 <sup>bc</sup>	71	12.014±0.575 <sup>c</sup>	60	7.162±0.399 <sup>b</sup>	8	4.111±0.857 <sup>b</sup>
	Water	75	1.8±0.1 <sup>c</sup>	71	12.962±0.574 <sup>c</sup>	57	7.207±0.438 <sup>b</sup>	4	4.440±1.404 <sup>b</sup>
30/30	10 <sup>-6</sup> M LCO	51	2.8±0.2 <sup>a</sup>	42	17.226±0.779 <sup>a</sup>	39	14.475±0.720 <sup>a</sup>	27	7.307±0.775 <sup>a</sup>
	10 <sup>-11</sup> M Thuricin 17	48	2.5±0.1 <sup>ab</sup>	43	13.659±0.903 <sup>ab</sup>	36	12.602±0.944 <sup>a</sup>	23	6.168±0.821 <sup>a</sup>
	Water	48	2.6±0.1 <sup>ab</sup>	39	17.258±0.769 <sup>a</sup>	38	14.292±0.934 <sup>a</sup>	19	7.896±0.917 <sup>a</sup>

\* superscripts indicate Dunn's test results ( $\alpha = 0.05$ ). Zero values and missing data points were eliminated for the calculation of means and standard errors

Table 4.2 Spearman correlation matrix for variables from the peat pellet assay																			
	T17	LCO	H <sub>2</sub> O	AboGroFr	AbvGroDr	PlnPerTr	PlnPerPl	HeigOfSc	HeiOfPr	Cotyledn	AdvnRoot	Stage	Tday	Leaves	LegOfLn	LengOfS	LengOfTh	LengOfFo	LengOfFi
T17	1	-0.5057	-0.49242	-0.09051	-0.08424	0.04663	-0.00384	0.0526	0.02251	0.05541	-0.06746	0.04018	0.00169	0.03451	-0.02187	-0.00487	-0.00075	-0.01379	-0.04105
LCO	-0.5057	1	-0.50185	0.06468	0.06164	0.00075	0.02197	0.01313	-0.05172	-0.05574	0.13243	-0.0376	0.00683	-0.02952	-0.01661	-0.01359	0.06588	0.04424	0.08117
H <sub>2</sub> O	-0.49242	-0.50185	1	0.02662	0.02326	-0.04751	-0.01831	-0.06628	0.02917	0.00068	-0.0663	-0.00235	-0.00859	-0.00483	0.03868	0.01859	-0.06571	-0.03081	-0.04073
AboGroFr	-0.09051	0.06468	0.02662	1	0.96799	-0.17937	0.01805	-0.01861	0.6154	0.0343	0.01846	0.86962	0.63511	0.85609	0.93364	0.86883	0.5098	0.24795	0.04874
AbvGroDr	-0.08424	0.06164	0.02326	0.96799	1	-0.16264	-0.00603	-0.02509	0.58676	0.01509	0.02703	0.85977	0.66863	0.86335	0.92515	0.87946	0.53062	0.27169	0.05433
PlnPerTr	0.04663	0.00075	-0.04751	-0.17937	-0.16264	1	0.07325	0.03029	-0.0615	0.3901	0.07894	0.10838	-0.10681	0.02165	0.01658	0.03327	0.04921	0.08946	-0.01762
PlnPerPl	-0.00384	0.02197	-0.01831	0.01805	-0.00603	0.07325	1	-0.02313	-0.07261	-0.13368	-0.05012	-0.08808	-0.01266	-0.06554	-0.0536	-0.05163	-0.02435	-0.05133	-0.01492
HeigOfSc	0.0526	0.01313	-0.06628	-0.01861	-0.02509	0.03029	-0.02313	1	0.09849	0.30391	-0.01669	0.08778	0.10838	0.09082	0.00052	-0.00355	0.10209	0.18111	0.31082
HeiOfPr	0.02251	-0.05172	0.02917	0.6154	0.58676	-0.0615	-0.07261	0.09849	1	0.43006	0.09192	0.69771	0.44731	0.65721	0.64352	0.56747	0.30769	0.14003	0.05044
Cotyledn	0.05541	-0.05574	0.00068	0.0343	0.01509	0.3901	-0.13368	0.30391	0.43006	1	0.07594	0.56312	0.03859	0.38406	0.33624	0.30391	0.19239	0.11796	0.08818
AdvnRoot	-0.06746	0.13243	-0.0663	0.01846	0.02703	0.07894	-0.05012	-0.01669	0.09192	0.07594	1	0.08846	0.06119	0.0871	0.04298	0.01601	0.08852	0.05157	0.22949
Stage	0.04018	-0.0376	-0.00235	0.86962	0.85977	0.10838	-0.08808	0.08778	0.69771	0.56312	0.08846	1	0.56468	0.95079	0.89253	0.87613	0.64147	0.32112	0.10315
Tday	0.00169	0.00683	-0.00859	0.63511	0.66863	-0.10681	-0.01266	0.10838	0.44731	0.03859	0.06119	0.56468	1	0.64534	0.60863	0.6087	0.46314	0.25192	0.08243
Leaves	0.03451	-0.02952	-0.00483	0.85609	0.86335	0.02165	-0.06554	0.09082	0.65721	0.38406	0.0871	0.95079	0.64534	1	0.931	0.9155	0.66507	0.33498	0.10743
LegOfLn	-0.02187	-0.01661	0.03868	0.93364	0.92515	0.01658	-0.0536	0.00052	0.64352	0.33624	0.04298	0.89253	0.60863	0.931	1	0.93211	0.53932	0.2533	0.05568
LengOfS	-0.00487	-0.01359	0.01859	0.86883	0.87946	0.03327	-0.05163	-0.00355	0.56747	0.30391	0.01601	0.87613	0.6087	0.9155	0.93211	1	0.58982	0.28059	0.06819
LengOfTh	-0.00075	0.06588	-0.06571	0.5098	0.53062	0.04921	-0.02435	0.10209	0.30769	0.19239	0.08852	0.64147	0.46314	0.66507	0.53932	0.58982	1	0.46528	0.14645
LengOfFo	-0.01379	0.04424	-0.03081	0.24795	0.27169	0.08946	-0.05133	0.18111	0.14003	0.11796	0.05157	0.32112	0.25192	0.33498	0.2533	0.28059	0.46528	1	0.31349
LengOfFi	-0.04105	0.08117	-0.04073	0.04874	0.05433	-0.01762	-0.01492	0.31082	0.05044	0.08818	0.22949	0.10315	0.08243	0.10743	0.05568	0.06819	0.14645	0.31349	1

**Table 4.3 Eigenvectors based on ranked variables and Spearman correlations from *B. napus* plants grown for 38 d at 10/4 °C in plant culture vessels**

	PC1	PC2	Polycot	T17A	T17B
NaCl concentration	-0.3196	0.14976	-0.0338	-0.1127	0.02321
Thuricin 17 concentration	-0.0019	0.05347	0.02742	0.51111	0.84217
Secondary stem height	0.14064	0.42982	0.06994	-0.095	-0.0584
Number of cotyledons	0.20347	-0.0018	0.54832	-0.0085	-0.0744
Number of leaves	0.33072	-0.1072	-0.1473	-0.0322	-0.0293
Fresh weight	0.29009	-0.1227	0.23947	0.05285	0.04085
Dry weight	0.29366	-0.1314	0.09878	0.18277	-0.0436
Primary stem height	0.10297	-0.0351	0.56445	-0.2713	0.16563
Primary root length	0.32227	-0.1675	0.06379	0.02163	-0.0134
Number of plants	0.185	-0.0555	-0.2672	0.24209	-0.1539
Longest petiole length	0.32776	-0.1255	-0.0995	0.01899	-0.0263
Second-longest petiole length	0.32324	-0.111	-0.1171	-0.0287	-0.0175
Third-longest petiole length	0.27739	-0.0168	-0.2903	-0.1376	0.09627
Fourth-longest petiole length	0.21089	0.13132	-0.3093	-0.2717	0.21661
Fifth-longest petiole length	0.15839	0.3761	-0.0369	-0.3215	0.22115
Sixth-longest petiole length	0.15415	0.4066	-0.0014	-0.253	0.11748
Seventh-longest petiole length	0.11419	0.4251	0.04873	0.37796	-0.2298
Eighth-longest petiole length	0.11419	0.4251	0.04873	0.37796	-0.2298

<b>Table 4.4 <i>B. napus</i> cv. 04C111 plant architectural metrics, 20 DAP in plant culture vessels</b>					
NaCl ( $\times 10^{-3}$ M)	Thuricin 17 (M)	<i>n</i>	Primary stem height (mm $\pm$ se)	Leaf count $\pm$ se	Sum of petiole lengths (mm $\pm$ se)
0	0	45	$1.645 \pm 0.053^{a*}$	$3.0 \pm 0.2^{abc}$	$2.932 \pm 0.354^{ab}$
	$10^{-11}$	10	$1.602 \pm 0.133^{ab}$	$3.0 \pm 0.2^{abc}$	$2.129 \pm 0.226^{abc}$
	$10^{-9}$	31	$1.592 \pm 0.051^a$	$3.5 \pm 0.3^{ab}$	$4.182 \pm 0.696^a$
0.05	0	50	$1.659 \pm 0.068^a$	$3.4 \pm 0.2^{ab}$	$3.424 \pm 0.373^a$
	$10^{-11}$	12	$1.639 \pm 0.064^a$	$3.6 \pm 0.3^a$	$2.523 \pm 0.254^{ab}$
	$10^{-9}$	34	$1.659 \pm 0.040^a$	$3.5 \pm 0.2^a$	$3.863 \pm 0.494^a$
50	0	41	$1.406 \pm 0.053^{ab}$	$3.1 \pm 0.2^{abc}$	$2.778 \pm 0.343^{ab}$
	$10^{-11}$	13	$1.479 \pm 0.047^{ab}$	$3.1 \pm 0.2^{abc}$	$2.040 \pm 0.159^{abc}$
	$10^{-9}$	34	$1.476 \pm 0.063^{ab}$	$2.8 \pm 0.3^{abc}$	$2.879 \pm 0.445^{abc}$
100	0	35	$1.157 \pm 0.038^b$	$2.1 \pm 0.2^{bc}$	$1.077 \pm 0.140^c$
	$10^{-11}$	13	$1.132 \pm 0.073^b$	$2.2 \pm 0.3^{abc}$	$1.484 \pm 0.243^{abc}$
	$10^{-9}$	26	$1.198 \pm 0.058^b$	$1.8 \pm 0.3^c$	$1.037 \pm 0.259^b$

\* superscript letters indicate differences,  $p < 0.05$  (Saxton 1998).

**Table 4.5 Eigenvectors based on ranked variables and Spearman correlations from *B. napus* plants grown for 20 d at continuous 30 °C in plant culture vessels**

	PC1	Radial symmetry	Secondary embryogenesis	NaCl	T17
Thuricin 17 concentration	0.00337	0.00836	0.0181	-0.2654	0.84445
NaCl concentration	-0.1704	0.08827	-0.3541	0.54461	0.0447
Secondary stem height	0.00352	0.096	0.57531	0.23867	-0.2493
Number of cotyledons	0.00206	0.25945	0.51418	0.21108	0.13438
Number of plants	-0.0377	0.24302	0.37561	0.11944	0.30738
Number of leaves	0.39158	-0.1009	0.01144	0.1851	-0.0031
Primary stem height	0.21145	-0.1447	0.2641	-0.457	-0.1981
Longest petiole length	0.38293	-0.1842	0.01327	-0.0886	0.02695
Second-longest petiole length	0.3851	-0.209	-0.0054	-0.0409	0.05263
Third-longest petiole length	0.38253	-0.1339	-0.0114	0.18407	0.0512
Fourth-longest petiole length	0.36351	-0.0408	0.00669	0.27381	0.01618
Fifth-longest petiole length	0.30647	0.17422	-0.1203	0.21518	0.09749
Sixth-longest petiole length	0.23403	0.41352	-0.1472	0.0262	0.08923
Seventh-longest petiole length	0.17604	0.52414	-0.1439	-0.2072	-0.1341
Eighth-longest petiole length	0.13556	0.50213	-0.1038	-0.2565	-0.1708

## 4.8 Figure captions

**Figure 4.1** Mean lengths of petioles produced by *B. napus* cv. 04C111 grown in peat pellets for 11 days under a range of temperature conditions. Treatments: distilled water control (○),  $10^{-6}$  M solution of lipo-chitooligosaccharide (+), and  $10^{-11}$  M solution of thuricin 17 (×). Error bars indicate upper and lower quantiles.

**Figure 4.2** First (x-axis) and second (y-axis) principal components: AboGroFr = above ground fresh weight; AbvGroDr = above ground dry weight; AdvnRoot = presence of adventitious root; Cotyledn = number of cotyledons; H<sub>2</sub>O = control treatment; HeigOfSc = height of second stem; HeiOfPr = height of primary stem; LCO = LCO treatment; Leaves = number of leaves; LegOfLn = length of the longest petiole; LengOfTh = length of the third-longest petiole; LengOfFi = length of the fifth-longest petiole; LengOfFo = length of the fourth longest petiole; LengOfSc = length of the second-longest petiole; PlnPerPl = plants per peat pellet; PlnPerTr = plants per tray; Stage = plant developmental stage (11 days of growth); T17 = thuricin 17 treatment; Tday = daytime temperature.

**Figure 4.3** First (x-axis) and third (y-axis) principal components: AboGroFr = above ground fresh weight; AbvGroDr = above ground dry weight; AdvnRoot = presence of adventitious root; Cotyledn = number of cotyledons; H<sub>2</sub>O = control treatment; HeigOfSc = height of second stem; HeiOfPr = height of primary stem; LCO = LCO treatment; Leaves = number of leaves; LegOfLn = length of the longest petiole; LengOfTh = length of the third-longest petiole; LengOfFi = length of the fifth-longest petiole; LengOfFo = length of the fourth longest petiole; LengOfSc = length of the second-longest petiole; PlnPerPl = plants per peat pellet; PlnPerTr = plants per tray; Stage = plant developmental stage (11 days of growth); T17 = thuricin 17 treatment; Tday = daytime temperature.

**Figure 4.4** A 3-D surface plot of above-ground fresh weight of Topas plants grown for 2 weeks. Agfw = above ground fresh weight (g), conc = concentration of LCO ( $10^x$  M), temp = temperature (°C).

**Figure 4.5** A 3-D surface plot of above-ground dry weight of Topas plants grown for 2 weeks. Agdw = above ground dry weight (g), conc = concentration of LCO ( $10^x$  M), temp = temperature ( $^{\circ}$ C).

**Figure 4.6** A 3-D surface plot of plant developmental stages achieved by Topas plants grown for 2 weeks. Stage = plant developmental stage (Sylvester-Bradley 1985), conc = concentration of LCO ( $10^x$  M), temp = temperature ( $^{\circ}$ C).

**Figure 4.7** Stem heights of 04C111 plants grown for 20 days in plant culture vessels under 24 h 30  $^{\circ}$ C. Bars indicate standard errors. Letters indicate difference,  $\alpha = 0.05$  (Saxton 1998).

**Figure 4.8** Numbers of leaves produced by 04C111 plants grown for 20 days in plant culture vessels under 24 h 30  $^{\circ}$ C. Bars indicate standard errors. Letters indicate difference,  $\alpha = 0.05$  (Saxton 1998).

**Figure 4.9** Sums of lengths of petioles produced by 04C111 plants grown for 20 days in plant culture vessels under 24 h 30  $^{\circ}$ C. Bars indicate standard errors. Letters indicate difference,  $\alpha = 0.05$  (Saxton 1998).

**Figure 4.10** Mean lengths of petioles produced by 04C111 plants grown for 20 days in plant culture vessels at 24 h 30  $^{\circ}$ C and treated with a range of NaCl concentrations.  $10^{-11}$  M thuricin 17 ( $\circ$ ),  $10^{-9}$  M thuricin 17 (+), water ( $\times$ ). Error bars indicate the upper and lower quartiles.

## 4.9 Figures

Figure 4.1

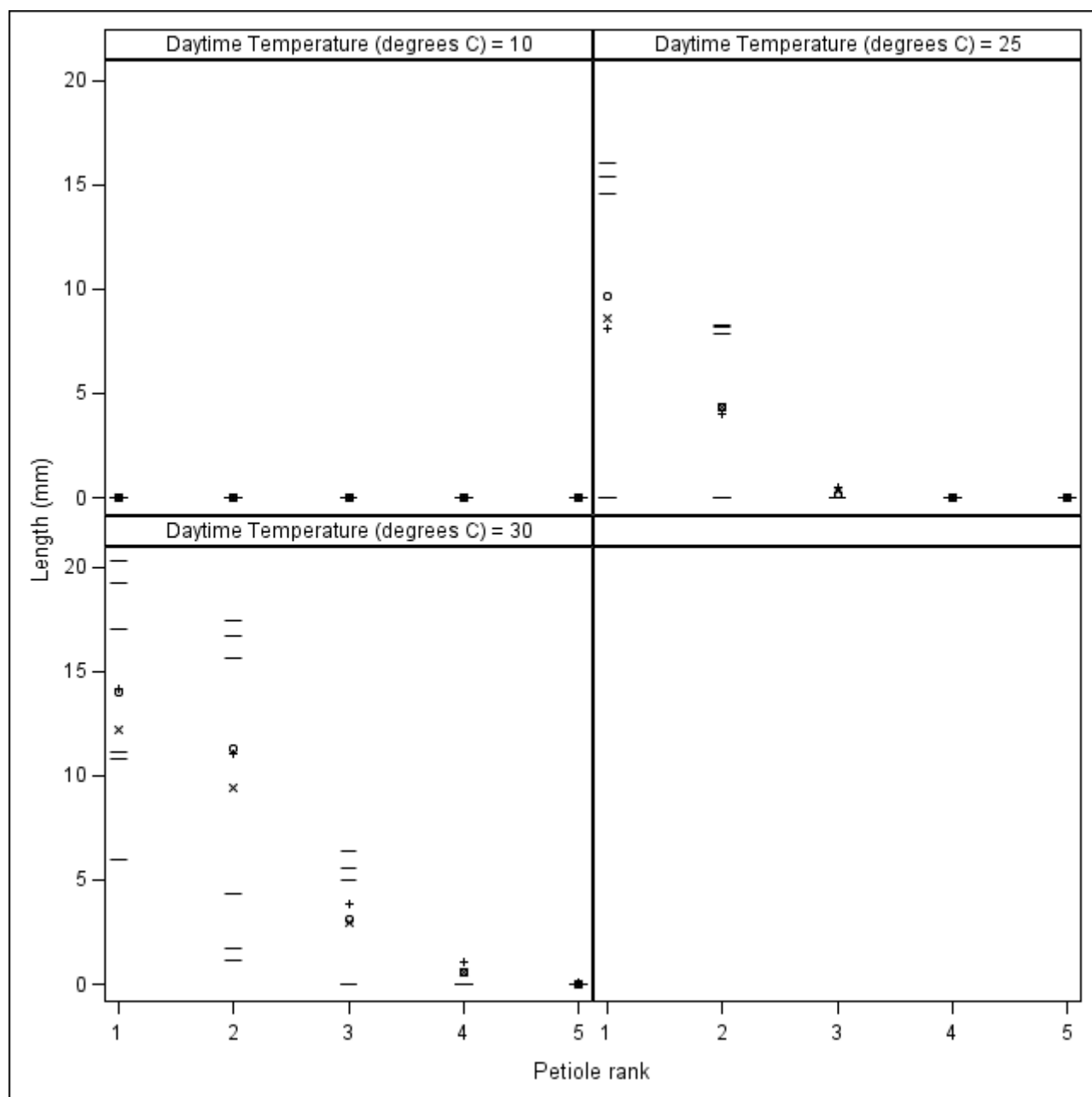


Figure 4.2

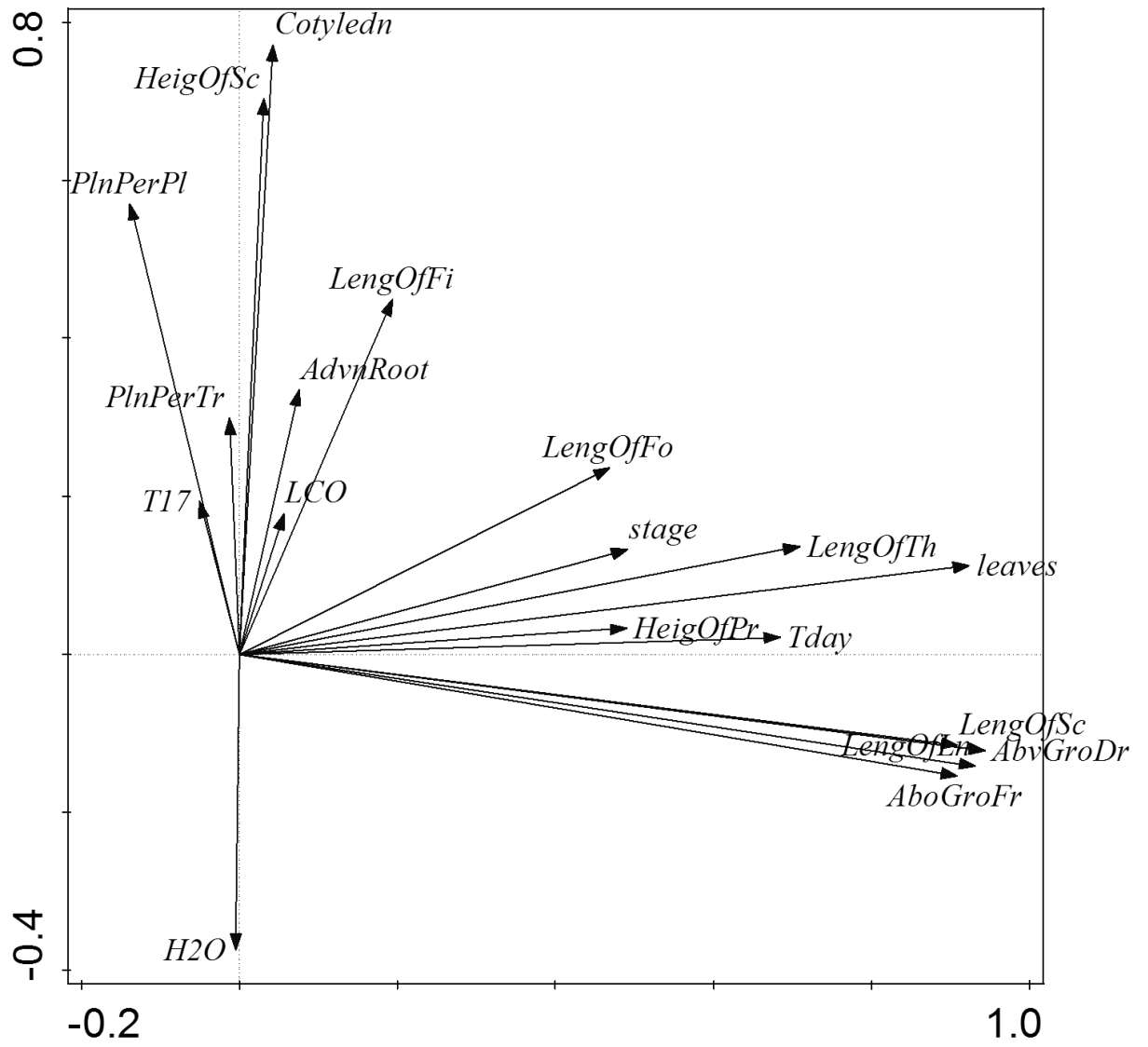


Figure 4.3

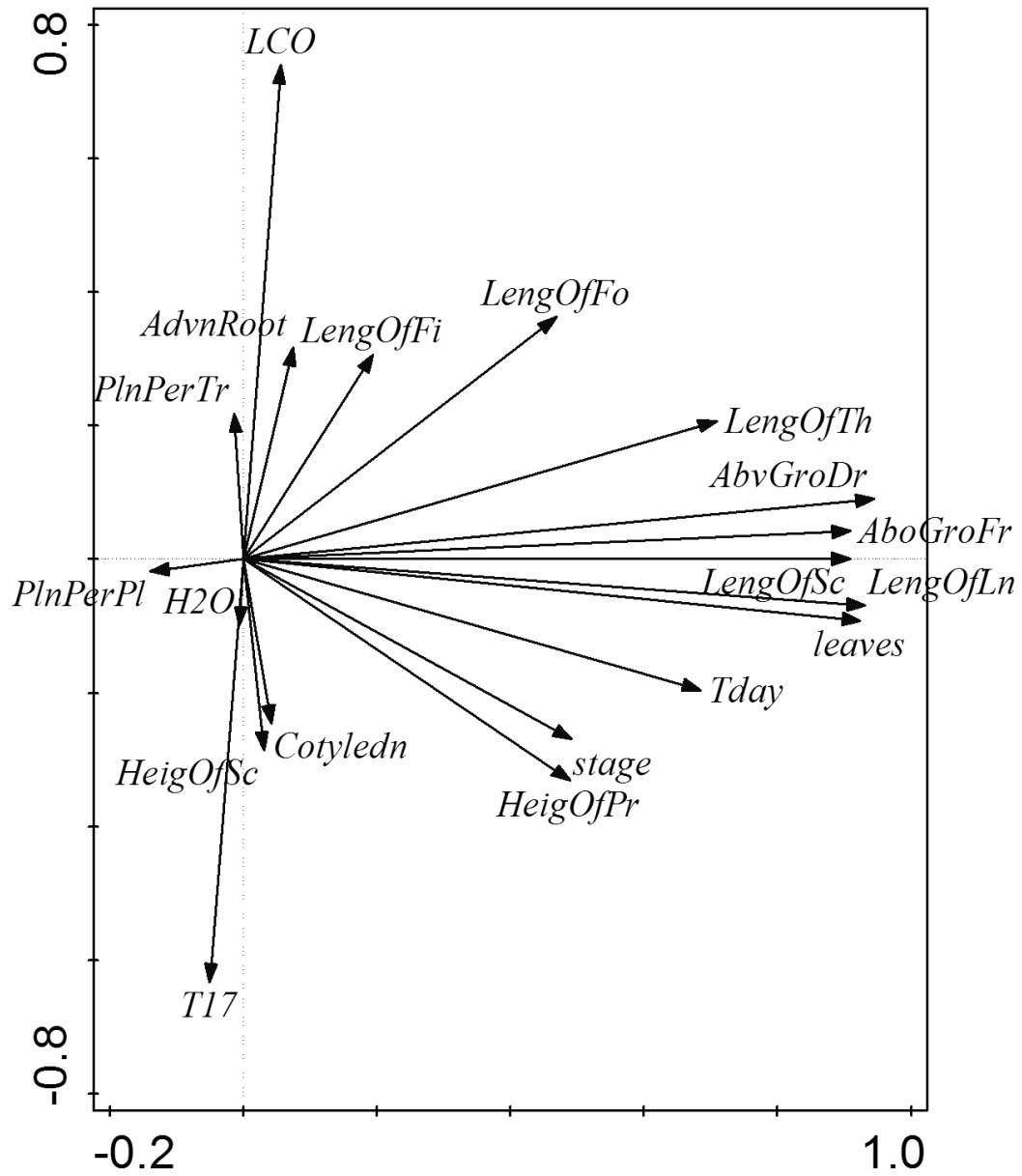


Figure 4.4

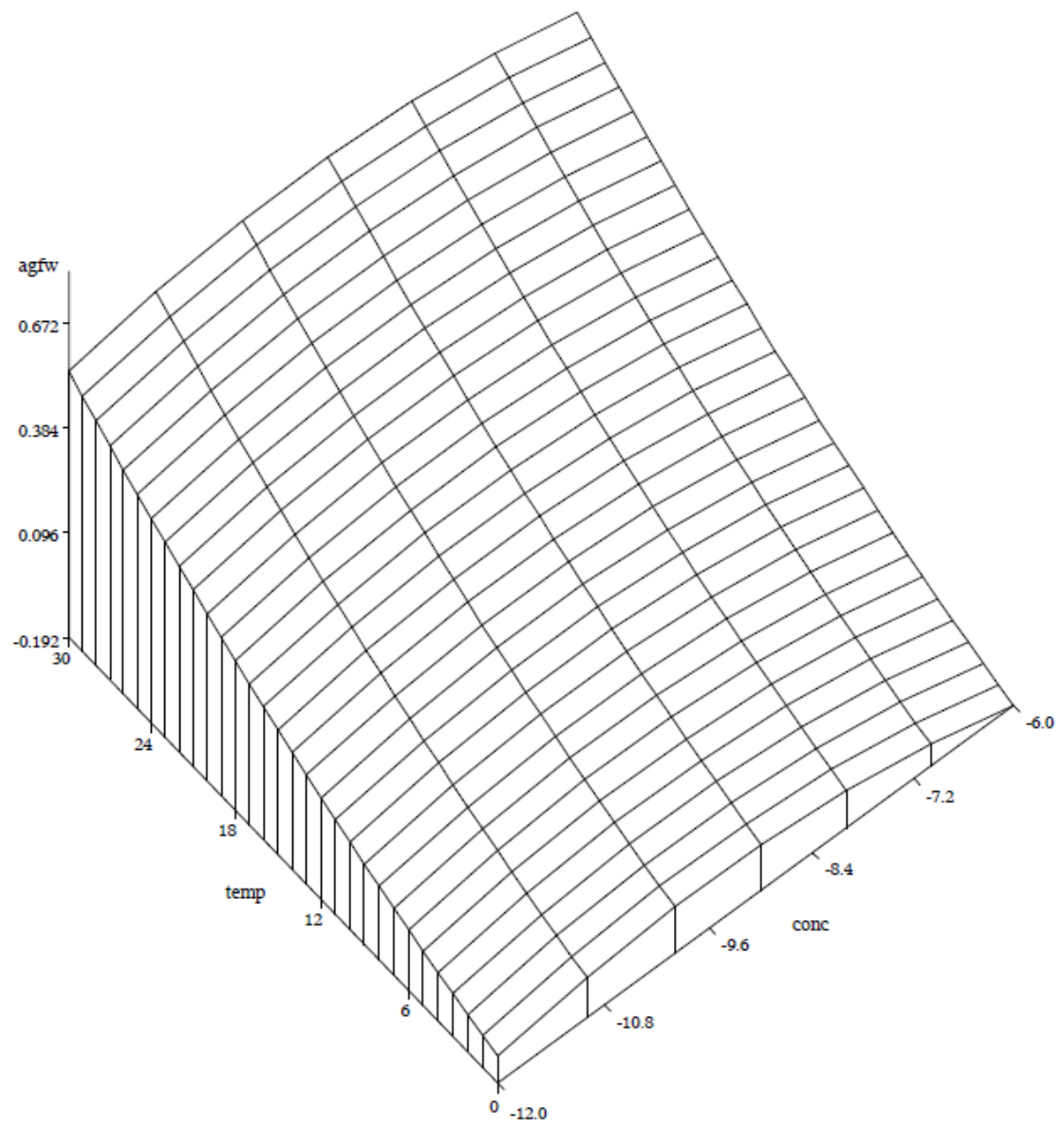


Figure 4.5

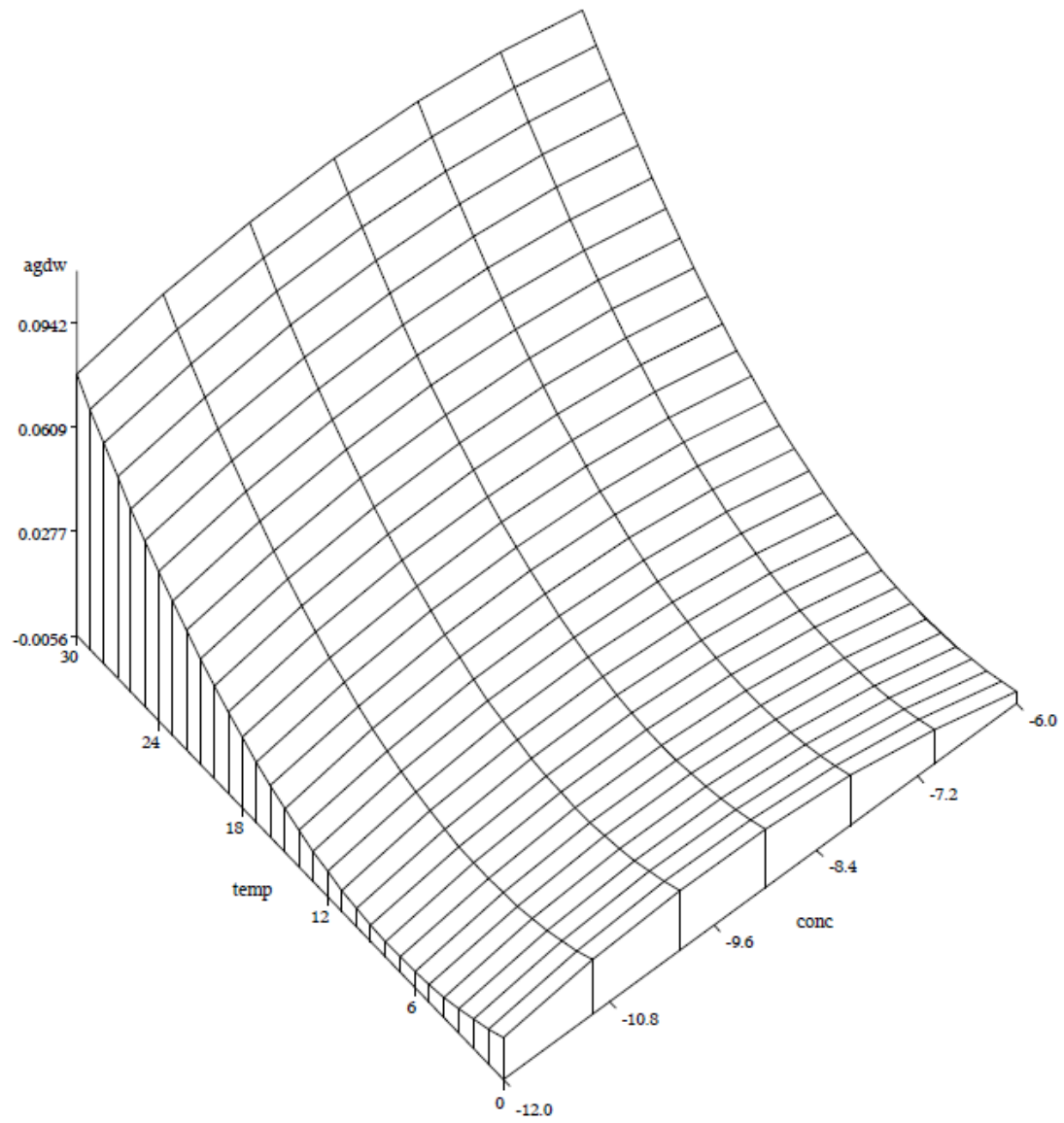


Figure 4.6

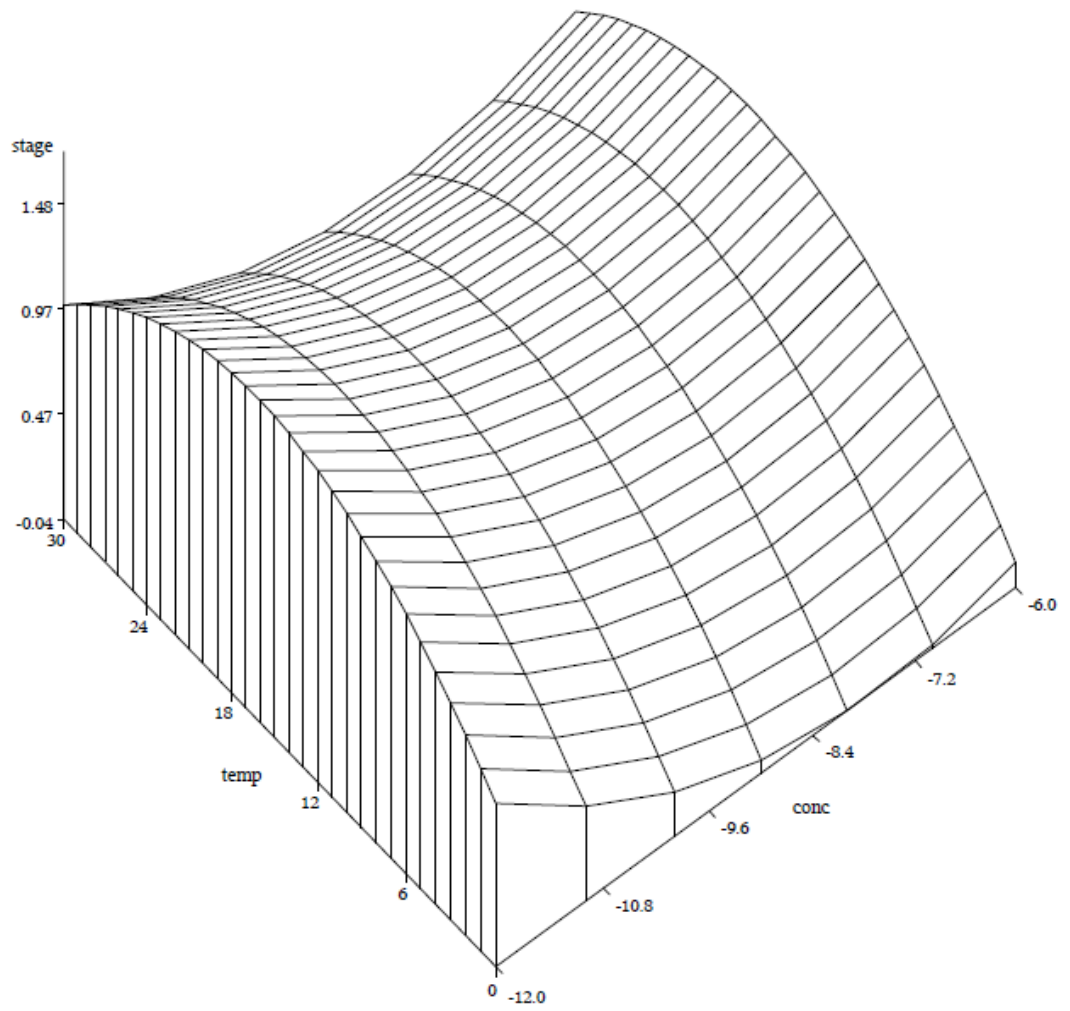


Figure 4.7

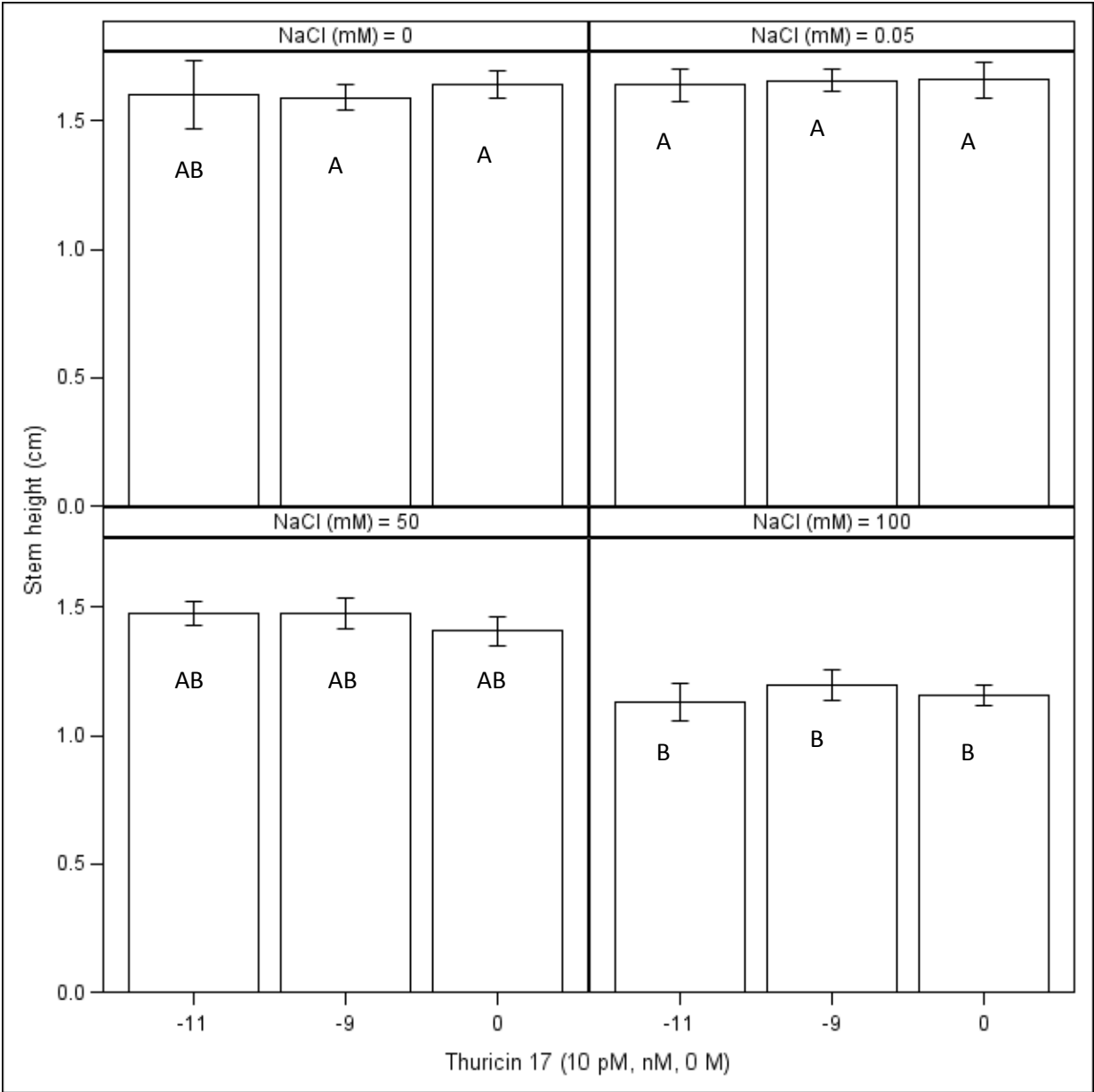


Figure 4.8

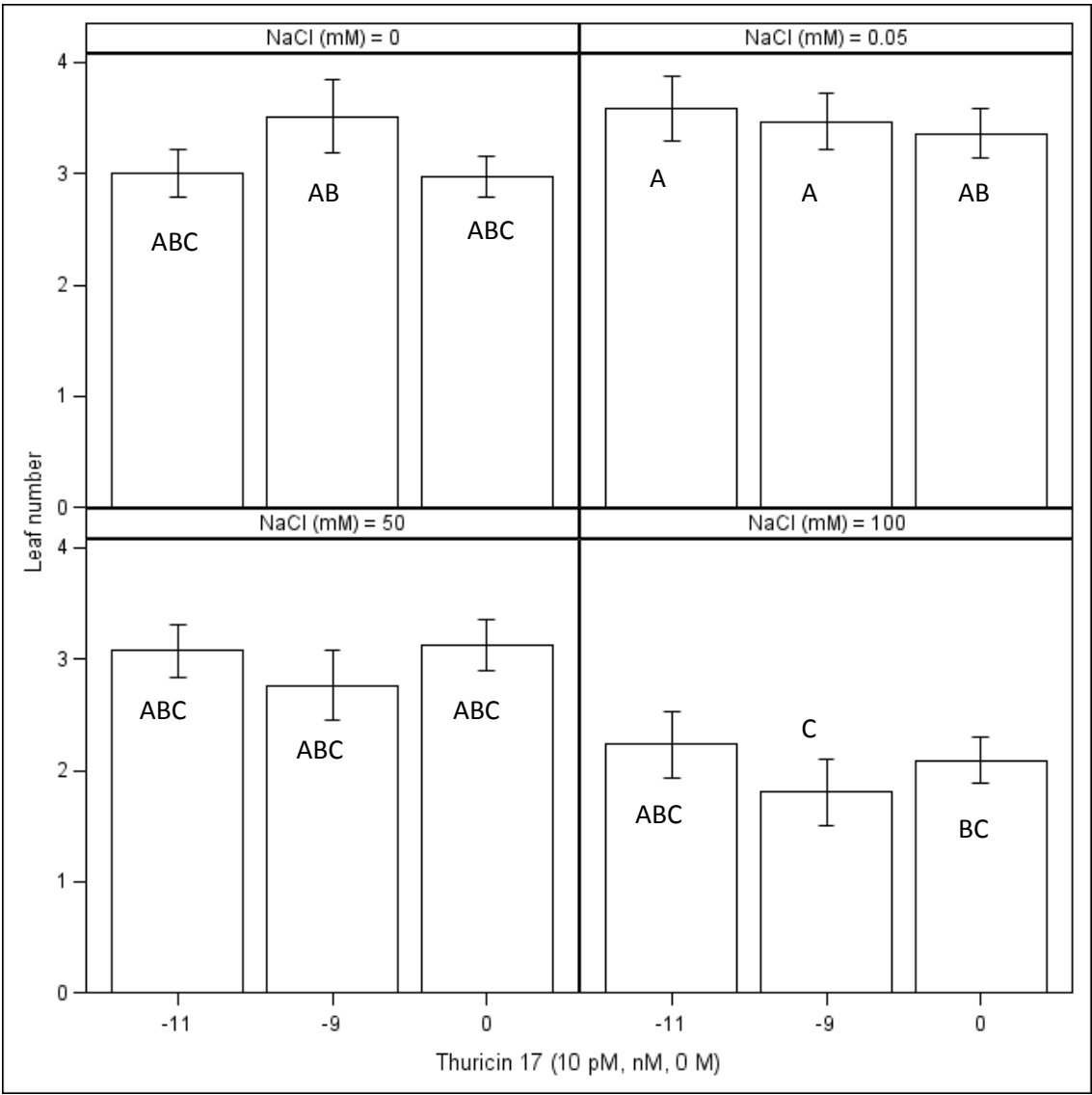


Figure 4.9

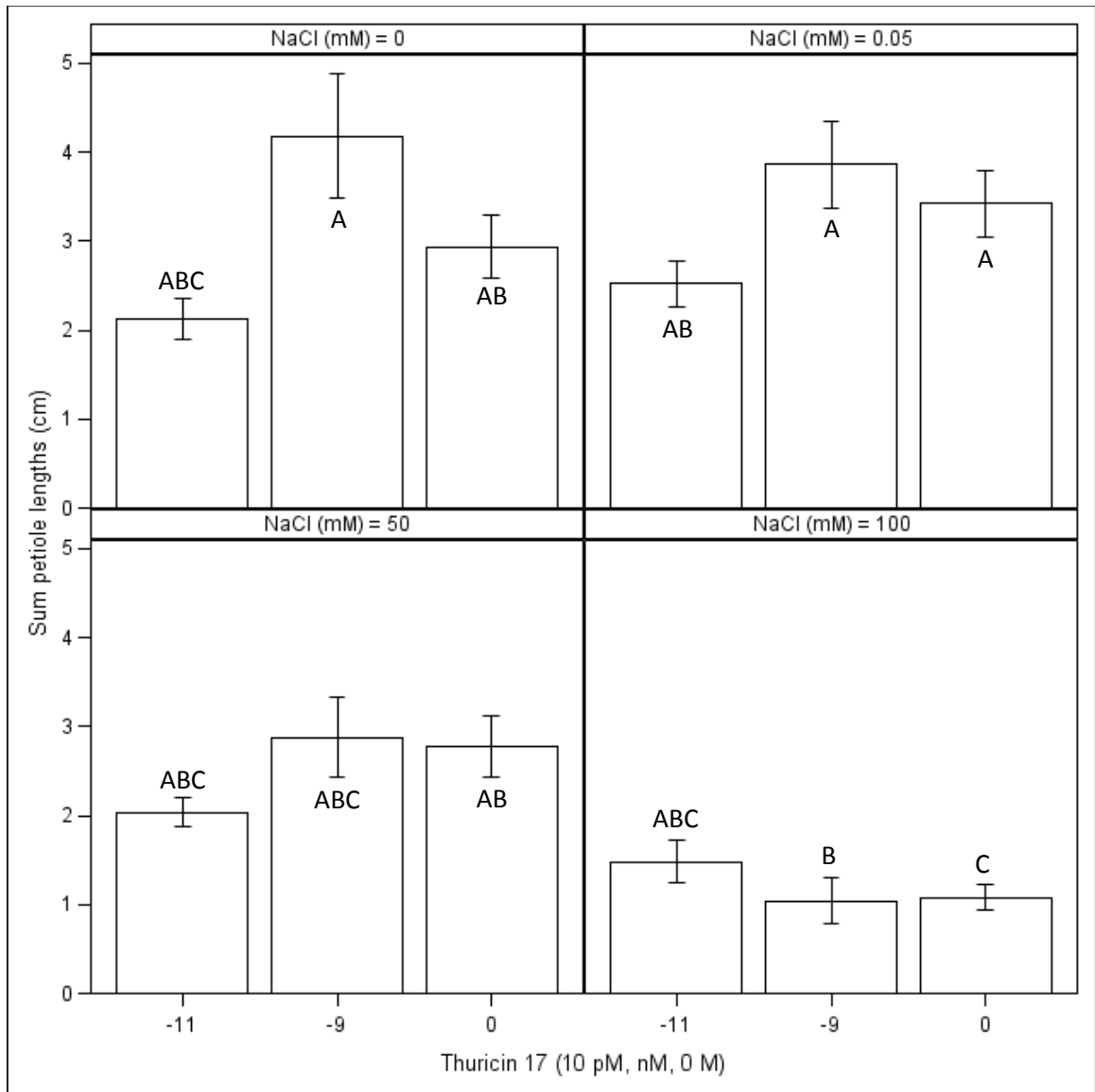
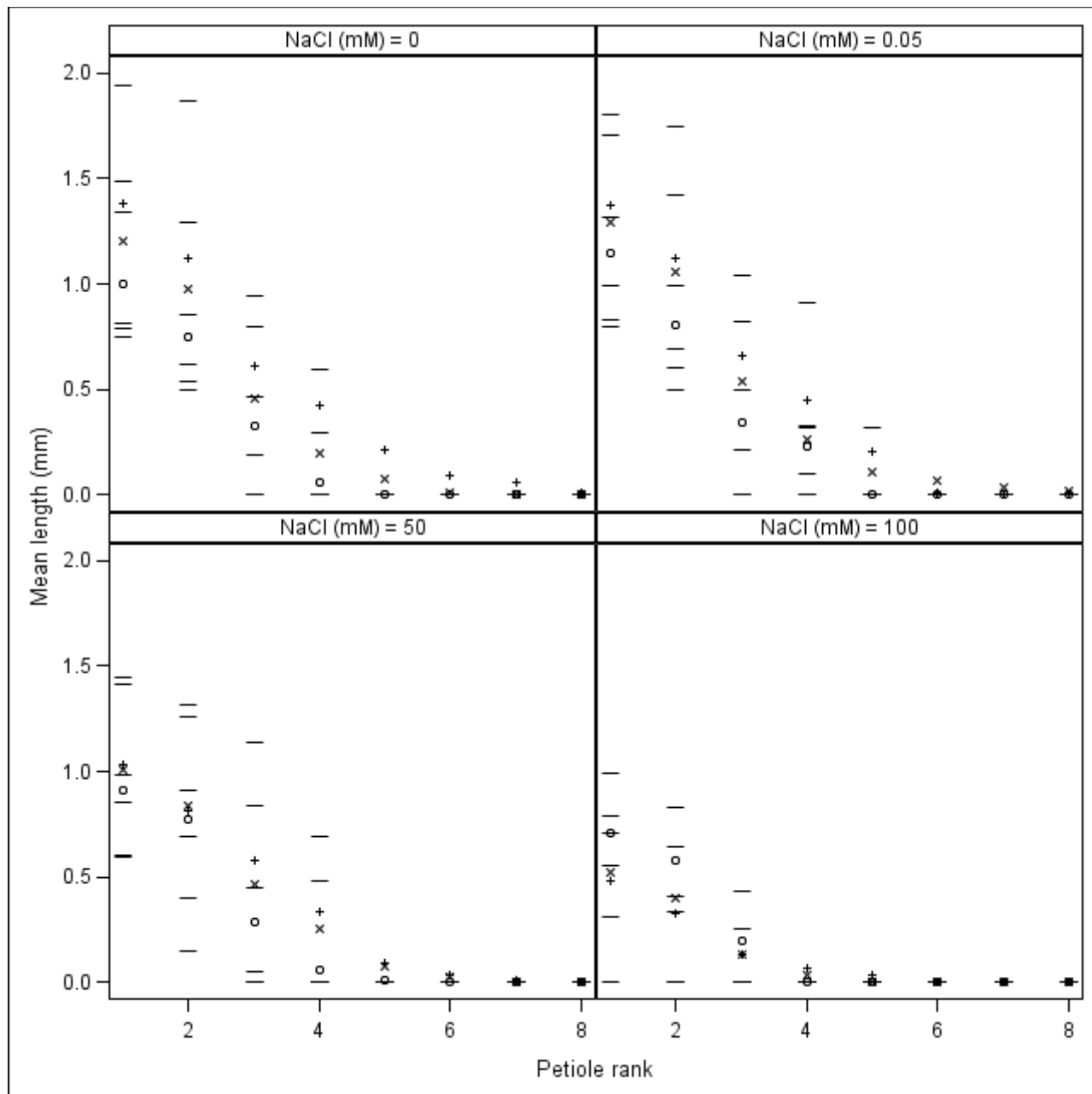


Figure 4.10



## 5 Preface to Section 5

Section 5 is a manuscript by Schwinghamer, Souleimanov, Dutilleul, and Smith that will be submitted to Biomass & Bioenergy. The literature cited in this section is listed in the reference section (page 160). Each figure caption, figure, and table is presented at the end of the section.

This section demonstrates the potential of an acute treatment of  $10^{-6}$  M LCO at planting to enhance the emergence, seed production, and seed weight of rapid cycling *B. napus* fastplants. The potential role for the treatment to enhance plant growth as a foliar spray is addressed.

The thesis consists of 4 papers: the first focused on the beginning of development, *i.e.*, germination, and the second focused on subsequent growth and early development. This is the third, which focuses on development right through to yield, but using a rapid system. The fourth will focus on development, including plant architecture through to yield with conventional genotypes.

## **A micromolar concentration of lipo-chitooligosaccharide (Nod B<sub>j</sub> V [C18:1, MeFuc]) regulates the emergence and seed productivity of rapid cycling canola (*Brassica napus* [L.] plants**

### **5.1 Abstract**

Canola (*Brassica napus* L.) seed set is reduced under high temperature conditions, resulting in yield reductions under field conditions. We saw previously that LCO treatments and irrigation with LCO produce relatively multifoliate young *B. napus* cv. 04C111 plants under moderately high temperature conditions. The objective of this experiment was to assess whether or not the application of 10 mL 10<sup>-6</sup> M LCO to rapid cycling *B. napus* seed, and the application of a supplemental 10<sup>-6</sup> M LCO foliar spray, would increase factors that contribute to yield under moderately high (24 h 25 °C) temperature conditions. Rapid cycling *B. napus* plants were grown to maturity in controlled environment cabinets to assess the effects of a 10<sup>-6</sup> M LCO solution applied to the seed at planting and with a supplementary foliar spray. Application of LCO to the seed at planting accelerated rapid cycling plant emergence and increased the number of emerged plants by 28.1 %, one week after planting. The application of LCO to the seed at planting also increased the number of plants that produced seed by 15.4 %, and increased total seed weight produced from mature plants by 2.009 ± 0.561 mg ( $p_{\text{diff}} = 0.0295$ ).

### **5.2 Introduction**

*Brassica napus* is described as having origins in the Mediterranean region, and it may have several centres of evolution (Tsunoda 1980). Brassicaceous plants have provided fuel since at least the 5<sup>th</sup> century BC, and possibly since the 7<sup>th</sup> century BC, during the Roman age of Egypt (Colombini et al. 2005). Throughout the Middle Ages in Europe, lamps fuelled by rapeseed oil were ubiquitous (Dixon 2007). *Brassica napus* declined in agricultural popularity after the proliferation of fossil fuels, but the cultivated area of *B. napus* has expanded in recent decades, in part owing to the intensifying demand for biodiesel feedstock (Franzaring et al. 2008). Appropriate field rotations, which include *B. napus* for sustainable food and fuel production in Europe, were reviewed by Zegada-Lizarazu and Monti (2011).

Although *B. napus* has been known to science at least since the 19<sup>th</sup> century, scientific research on *B. napus* began in the late 1930s (Juska and Busch 1994). At that time, many countries developed national policies to promote self-sufficiency in production

of fats and oils, and such concerns are still current present in much of the world. In the early 1970s, responding to health concerns, Canadian breeders produced varieties with less than two percent erucic acid in the oil and less than  $30 \times 10^{-6}$  M of glucosinolates per gram of air-dried oil-free meal. In 1979, these new varieties were given the name “canola,” *i.e.*, Canadian oil low acid, also known as edible oilseed rape. Rapid cycling *B. napus* was developed as an efficient and inexpensive tool for education and research (Williams and Hill 1986). The rapid cycling *Brassicas* are capable of producing ten or more generations per year (Aslam et al. 1990). Ergo, similar to *Arabidopsis thaliana* [L.] Heynh., rapid cycling *B. napus* does not require a field season to provide results. The criteria used in selection for their breeding were: minimum time to flowering, rapid seed maturation, absence of seed dormancy, smallness, and therefore only a limited number of flower buds, but high female fertility (Williams and Hill 1986).

In agricultural production experiments, high temperature conditions at the flowering stage of plant development have long been observed to reduce *B. napus* yields (*e.g.*, Johnson et al. 1995), and the critical temperature was determined by later workers. Morrison (1993) reported *B. napus* cvs. Westar and Delta grown at a daily mean 22°C in growth cabinets were almost entirely sterile, and abnormal megagametophyte development, owing to high temperature stress, has been observed among *B. napus* plants at a daily mean 25.5 °C (Polowick and Sawhney 1987). Thus, new plant growth regulators that aid *B. napus* in withstanding heat stress during flowering could have important applications in *B. napus* research, breeding, and agriculture.

Nod factors are LCOs excreted by rhizobia as signals to their legume or *Parasponia* hosts. The AM fungus *Glomus intraradices* also secretes symbiotic sulphated and non-sulphated simple LCOs, which stimulate the formation of AM symbiosis in plant species of diverse families: *Fabaceae*, *Asteraceae*, and *Umbelliferae* (Maillet et al. 2011). Preliminary analysis suggested that the uncultured Dg1 symbiont of Durango root (*Datisca glomerata* [C. Presl] Baill.) contains *nodABC*-like genes, which suggests that it uses Nod factor-like compounds during the infection process (Persson et al. 2011). The molecule's backbone consists of five  $\beta$ -1,4-linked *N*-acetyl-D-glucosaminyl (GlcNAc) residues plus an *N*-acyl chain at the non-reducing terminus. LCOs may represent a novel class of plant growth regulator involved in plant developmental processes (Dénarié and Cullimore 1993). Dyachok et al. (2002) found

that Nod factors, and LCOs analogous to rhizobial Nod factors, suppress cell death and occur in embryogenic cultures of Norway spruce (*Picea abies* [L.] Karst.). They found relatively high concentrations of LCO among pro-embryogenic cell masses indicative of LCO's stimulation of early processes during somatic embryogenesis.

In *Arabidopsis*, lysin motif proteins have been shown to bind GlcNAc residues (Ohnuma et al. 2008). LysM domains associated with a receptor-like kinase domain form a LysM-RLK, termed CERK1 or LysM-RLK1 (Miya et al. 2007; Wan et al. 2008), that binds insoluble chitin and binds chitooligosaccharide weakly (Iizasa et al. 2010; Petutschnig et al. 2010). Recently published work indicated that a smaller brassicaceous plant, *Arabidopsis*, responded to LCO treatment by reducing levels of FLAGELLIN-SENSING 2 (FLS2) receptors located on the plasma membrane, and suppressing its microbial-associated molecular pattern (MAMP) -triggered reactive oxygen species (ROS) production (Liang et al. 2013).

Our own laboratory has found that LCO can increase total abscisic acid (10.19 %) and salicylic acid (15.00 %), and decrease indolylacetic acid (-49.68 %), cytokinin (-36.24 %), gibberellic acid (-19.41 %), and jasmonic acid (-33.66 %) of *Arabidopsis* rosettes, 24 h post treatment (Subramanian, personal communication). These levels of salicylic acid accumulation are somewhat less than the 100 to 200 % increase over background levels that would be expected to be induced at the primary leaf due to second stage systemic acquired resistance (SAR) (Cameron et al. 1999). While SAR is associated with an increase in transcript levels of pathogenesis-related genes, pathogen response protein 1 was not found to increase in LCO-treated *Arabidopsis* (Subramanian, personal communication). The growth of diverse crop plants has been enhanced by LCO treatment (Zhang and Smith 2001; Prithiviraj et al. 2003). Previous experiments have shown interactions between cultivar and temperature conditions in the response of *B. napus* to LCO with regards to germination, and the architecture and biomass of young plants. However, whether or not they can enhance the yield of *B. napus* plants, testable through use of the very quickly developing rapid cycling *B. napus*, under moderately high temperature conditions is not known. The objective of this experiment was to assess the effects of a  $10^{-6}$  M LCO seed treatment, and the application of a supplemental  $10^{-6}$  M LCO foliar spray, on factors that contribute to the yield of rapid cycling *B. napus* plants.

## 5.3 Materials and methods

### 5.3.1 Experimental Methods

Lipo-chitooligosaccharide was produced as described in section 3.3.2. Rapid cycling *B. napus* seeds (ACaacc, ac = 19) (Brassica Genetics Cooperative, University of Wisconsin-Madison) were sown into small square (6.4 cm sides) pots (Kord Products, Toronto) of promix (Premier Horticulture Ltee, Rivière-du-Loup, QC, Canada) treated with 10 mL of  $\mu\text{M}$  LCO solution or distilled water, and fertilized with slow-release fertilizer (Nutricote 14-14-14; Plant Products Co. Ltd., Brampton, ON, Canada). The rapid cycling plants were grown under continuous light ( $250 \text{ mmol m}^{-2} \text{ s}^{-1}$ ) (Hansen and Earle 1994). The plants were grown at  $24 \text{ h } 25^\circ\text{C}$ , because this is the standard temperature for rapid cycling *B. napus* plant growth (Williams and Hill 1986). The plants were watered uniformly with deionized water, as necessary. Emergence data were taken after one week. Supplementary foliar sprays of  $10^{-6} \text{ M}$  LCO solution were applied with an atomizer (Nalgene USA) in sufficient quantity to coat the surface of the plant at successive stages of plant development ( $<1 \text{ mL plant}^{-1}$ ) (Table 5.1). When the plants had senesced, the siliquae were removed and dried in an oven at  $60^\circ\text{C}$  for three days. Seed weight per rapid cycling plant was measured by weighing together all the seeds from each plant using a Mettler AE-166 laboratory scale. Weight per seed was calculated by dividing the seed weight per plant by the number of seeds. The experiment was repeated three times and when the experiment was repeated the third time we included the seed-plus-spray treatment only at the flowering stage.

### 5.3.2 Statistical Analysis

The experiment was run three times for emergence data, but only twice for seed weight data, owing to faulty humidity controls for the controlled environment cabinets during one of the repetitions. The variance was homogenous across the three replications of the experiment and so datasets were pooled. We used SAS PROC GLIMMIX (version 9.2, SAS Institute Inc., Cary, NC, USA) to assess the emergence (young plants were either emerged or not) and productivity (plants either produced seed or did not) data that followed a binary distribution. Low Shapiro-Wilk statistics indicated that ANOVA residuals for seed number, seed weight per plant, and number of seeds per silique were not normally distributed (Shapiro and Wilk 1965). Therefore, p values for differences between treatments on seed number, seed weight per plant, mean individual seed weight, and numbers of seeds per silique were

obtained with Kruskal-Wallis nonparametric tests, using SAS PROC NPAR1WAY with the WILCOXON option, and adjustments for multiple comparisons were made using Dunn's Test (Elliot and Hynan 2010). Where the differences are significant, the mean ( $\mu$ ) and standard error (se) is reported. Spearman correlation coefficients ( $\rho$ ) between contributing factors to yield were calculated with SAS PROC CORR. To produce a holistic picture of rapid cycling plant yield, we used SAS PROC IML to obtain path coefficients ( $r$ ). We report results at the  $p < 0.1$  level and show  $p$  values in all cases where differences are reported.

## 5.4 Results

Treatment with  $10^{-6}$  M LCO solution applied to the seed at planting hastens the emergence of rapid cycling *B. napus* plants. Under the controlled environment conditions that we used, the treatment of seeds with  $\mu$ M LCO solution accelerated the emergence of young plants by 28.1 % ( $n_{\text{LCO}} = 142$ ,  $\mu = 79.6$  %,  $se = 3.4$ ;  $n_0 = 35$ ,  $\mu = 51.4$  %,  $se = 8.6$ ;  $p_{0\text{-LCO}} < 0.0010$ ). Mean individual seed weights from the first repetition of the experiment indicated that the optimal time for the application of  $10^{-6}$  M LCO solution as a foliar spray was the flowering stage, when stomatal conductance could be expected to peak. In subsequent replications of the experiment, the foliar spray was applied only during the flowering stage.

Treatment of seeds with a  $10^{-6}$  M LCO solution enhanced factors that contribute to the yield of rapid cycling *B. napus* plants. Seed treatment with 10 mL  $10^{-6}$  M LCO solution increased plant production of seeds by 15.4 %, compared to controls ( $n_{\text{LCO}} = 160$ ,  $\mu = 53.1$  %,  $se = 4.0$ ;  $n_0 = 69$ ,  $\mu = 37.7$  %,  $se = 5.9$ ;  $p_{0\text{-LCO}} = 0.0460$ ) (Fig. 5.1).  $T$ -tests indicated the difference between plants grown from untreated seed versus those grown from LCO-treated seed was significant ( $p = 0.0460$ ), and the difference between plants grown from untreated seed versus those grown from treated seed, that were subsequently sprayed, was also significant at the  $p < 0.1$  level ( $p = 0.0917$ ). Seed treatment did not affect the numbers of siliquae produced (only pods with seeds were counted). Although the rapid cycling *B. napus* plants were small and bore typically only 1 or 2 seeds per plant, seed treatment increased seed number by approximately 1 seed on a per plant basis ( $n_{\text{LCO}} = 160$ ,  $\mu = 2.1$  seeds,  $se = 0.2$ ;  $n_0 = 69$ ,  $\mu = 1.4$  seeds,  $se = 0.3$ ;  $p_{0\text{-LCO}} = 0.0504$ ). This contributed to increased seed weight per plant by 2.009 mg ( $n_{\text{LCO}} = 160$ ,  $\mu_{\text{LCO}} = 5.315$  mg,  $se = 0.561$ ;  $n_0 = 69$ ,  $\mu_0 = 3.306$  mg,  $se = 0.634$ ;  $p_{0\text{-LCO}} = 0.0295$ ). There were no differences in the factors that

contributed to yield, after adjustment for multiple comparisons among controls, seed-treated plants, and seed-plus-spray treated plants.

We omitted unproductive plants from our path analysis. The Spearman correlation coefficients revealed positive associations between yield (seed weight per plant) and each of the predictor variables: number of siliquae, seeds per silique, seeds per plant, and mean individual seed weight (Table 5.2). The analysis indicated that the number of seeds per plant was positively associated with yield ( $r = 0.978483$ ), and mean individual seed weight was positively but negligibly associated with yield ( $r = 0.0400791$ ). The numbers of seeds per silique was negatively associated with yield ( $r = -0.148279$ ), and the number of siliquae per plant was negatively but negligibly associated with yield ( $r = -0.001852$ ).

## 5.5 Discussion

Previous research has shown, seed yield for agricultural *B. napus* is determined by the duration of flowering (Johnston et al. 2002), but rapid cycling *B. napus* reaches maturity in only 40 days, and flowering lasted merely a week under the conditions described here. The diminutive plants produced correspondingly fewer blossoms. The correlation of plant size to flower production, for ordinary *B. napus*, has been noted by Mendham and Scott (1975). Flowering is widely acknowledged to be the most critical stage of *B. napus* development as a crop, when *B. napus* is most susceptible to stress (Richards and Thurling 1978). We expect high temperature conditions, such as a daily mean temperature of 25.5°C, to produce abnormal megagametophyte development and inhibit *B. napus* flower production, even in the turbulent, regulated cabinet environment (Polowick and Sawhney 1988; Omid et al. 2010). Many other research groups have found similar temperature conditions induce fruit abortion and have severe detrimental effects on seed productivity among agricultural *B. napus* cultivars (e.g., Polowick and Sawhney 1988; Young et al. 2004). Thus, treatments that aid *B. napus* in withstanding heat stress during flowering could have applications for agricultural production in warm climates.

Chen et al. (2007) identified that the flowering stage is a critical time for foliar application of LCO to stimulate fruiting. The foliar application to tomato (*Lycopersicon esculentum* Mill.) plants of 50 ng LCO per plant, applied during the

early flowering stage and the late flowering stage, accelerated flowering and fruiting, increased flower numbers, and improved fruit yield (Chen et al. 2007).

The application of a  $10^{-6}$  M LCO treatment to the seeds at planting enhanced emergence of the rapid cycling plants. Although the hormonal changes triggered by LCO in *Arabidopsis* rosettes, observed 24 h post-treatment by Subramanian (personal communication) were similar to SAR, we observed the treatment to elicit no hypersensitive response. That the effects of the LCO treatment lasted until senescence are not surprising, given that biological SAR was effective in *B. napus* for at least 3 weeks (Potlakayala 2006). As such, these results may benefit *B. napus* agriculture, as rapid and uniform emergence from the seedbed lead to the production of vigorous plants with high chlorophyll content in their leaves (Ghassemi-Golezani et al. 2008). High leaf chlorophyll content, stand uniformity, and fast and early crop canopy closure can provide weed-inhibiting light conditions at the soil surface to reduce herbicide inputs, reduce selection pressure for weed resistance, improve yield, improve quality, and increase agricultural *B. napus* profitability (Górski 1975; Silvertown 1980). Rapid early light interception also improves early biomass accumulation on an area basis, and this can also contribute to higher final yields (Gunasekera et al. 2006; Harker et al. 2012).

The works of other authors have shown repeatedly that the correlation between seed size and numbers of seeds per silique can be variable, even among ordinary cultivars of *B. napus*, and the variance of weight of mature dry seed is large (Austin 1972; Clarke 1979; Araneda-Durán et al. 2010). Other groups have identified at least three basic components of *B. napus* grain yield, *i.e.*, seed weight, siliques per plant, and seeds per silique (Fan et al. 2010). The correlation coefficients shown in Table 5.2 confirm Bybordi et al.'s (2010) finding of a correlation between seed number per plant and seed number per silique. Total lipid and protein content of *B. napus* seeds is correlated highly with seed size or weight (Borisjuk et al. 2013; Hu et al. 2013). Peltonen-Sainio and Jauhiainen (2008) also found negative correlation between seed weight and number. They suggest that this negative correlation may be emphasized in late maturing *B. napus* under northern growing conditions, where seed size is affected by the short growing season (Peltonen-Sainio and Jauhiainen 2008).

Other studies have revealed the effects of LCO treatments on other non-leguminous plants. For example, spraying leaves of field plants with submicromolar concentrations ( $10^{-6}$ ,  $10^{-8}$ , and  $10^{-10}$  M) of Nod factors caused a transient increase in the photosynthetic rates of *B. napus*, soybean (*Glycine max* [L.] Merr.), apple (*Malus domestica* [Borkh.]), rice (*Oryza sativa* [L.]), common bean (*Phaseolus vulgaris* [L.]), grape (*Vitis vinifera* [L.]), and maize (*Zea mays* [L.] ssp. *mays*) plants (Zhang and Smith 2001). Khan et al. (2008) also found that the foliar application of LCO enhanced the photosynthetic rate, leaf area, and dry matter of *Z. mays*. The reported increases in photosynthesis rates, measured by gas exchange rate using an open-system photosynthesis meter, were 36, 23, and 12 % from plants treated with  $10^{-6}$ ,  $10^{-8}$ , and  $10^{-10}$  M, respectively.

## 5.6 Conclusions

In conclusion, after one week of growth at 25 °C, a relatively high temperature that is prohibitive to seed set, seed treatment with 10 mL  $10^{-6}$  M LCO solution enhanced emergence by 28.1 % for rapid cycling *B. napus* plants. Seed treatment with 10 mL  $10^{-6}$  M LCO solution also increased, by 15.4 %, the proportion of plants that produced seeds and increased the yield per plant by 2.009 mg. Greenhouse experiments will be necessary to ascertain whether or not this treatment could increase the productivity of agricultural cultivars.

## 5.7 Tables

**Table 5.1** Treatments applied to *B. napus* fastplants

Used during repetition	Seed treatment (mL $\mu$ M LCO)	Foliar spray (mL $\mu$ M LCO)	Weeks elapsed after planting / plant developmental stage at final treatment
1,2,3	0	0	N/A
1,2,3	10	0	0 / dry seed
1	10	0.1	1 / leaf production
1	10	2.5	2 / stem extension
1	10	0.75	3 / flower bud development
1,3	10	0.5	4 / flowering
1	10	0.5	5 / pod development

**Table 5.2 The Spearman's correlation coefficients for factors that contribute to yield produced by rapid cycling *B. napus***

Treatment	Yield component (plant <sup>-1</sup> )	Seed weight	Number of siliquae	Seeds per siliqua	Number of seeds	Mean individual seed weight
Seeds treated with 10 ml water	Seed weight	1.00000	0.50931**	0.52825**	0.74948***	0.19015
	Number of siliquae	0.50931**	1.00000	-0.14883	0.39573*	0.03472
	Seeds per siliqua	0.52825**	-0.14883	1.00000	0.79463***	-0.42295*
	Number of seeds	0.74948***	0.39573*	0.79463***	1.00000	-0.41462*
	Mean individual seed weight	0.19015	0.03472	-0.42295*	-0.41462*	1.00000
Seeds treated with 10 ml 1 µM LCO	Seed weight	1.00000	0.34466*	0.67239***	0.84336***	0.23257
	Number of siliquae	0.34466*	1.00000	-0.13584	0.33806*	0.16467
	Seeds per siliqua	0.67239***	-0.13584	1.00000	0.85983***	-0.42536**
	Number of seeds	0.84336***	0.33806*	0.85983***	1.00000	-0.27603
	Mean individual seed weight	0.23257	0.16467	-0.42536**	-0.27603	1.00000
Seeds treated with 10 ml 1 µM LCO and 1 µM LCO foliar spray at flowering	Seed weight	1.00000	0.22402	0.80893***	0.87268***	0.18580
	Number of siliquae	0.22402	1.00000	-0.16630	0.26044	0.15353
	Seeds per siliqua	0.80893***	-0.16630	1.00000	0.88877***	-0.25194
	Number of seeds	0.87268***	0.26044	0.88877***	1.00000	-0.24765
	Mean individual seed weight	0.18580	0.15353	-0.25194	-0.24765	1.00000

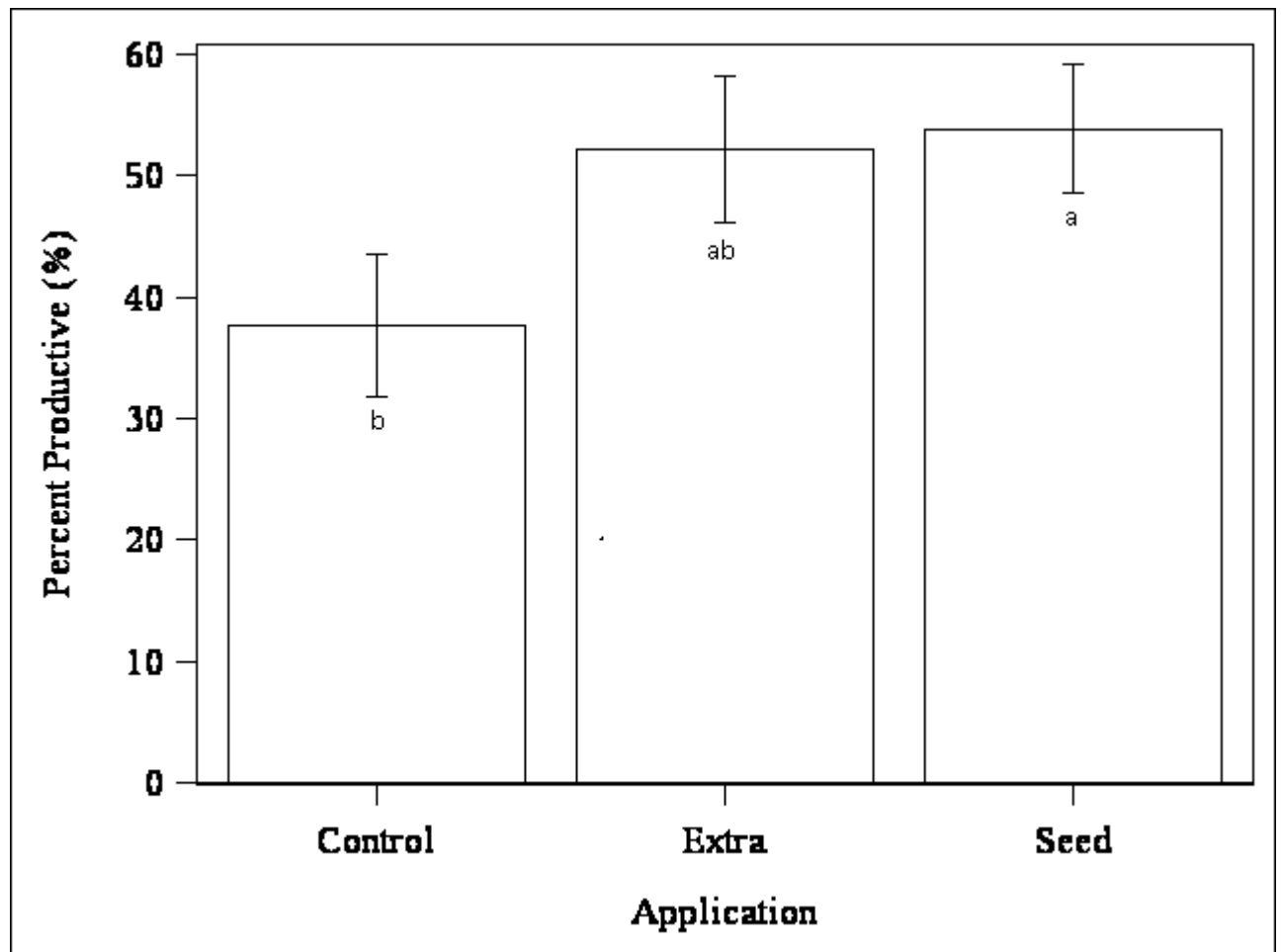
\*\*\* p < 0.0001; \*\* p < 0.01; \* p < 0.05

## 5.8 Figure captions

**Figure 5.1** Effect of treatment with  $\mu\text{M}$  LCO solution on the proportion of *B. napus* rapid cycling plants producing seeds. 1- untreated plants; 2- seed-treated with 10 mL  $\mu\text{M}$  LCO solution; 3- seed-treated with 10 mL  $\mu\text{M}$  LCO solution and leaves sprayed with 0.5 mL  $\mu\text{M}$  LCO solution at the flowering stage. Lower case letters indicate difference,  $p < 0.05$ . Bars indicate standard errors.

## 5.9 Figures

Figure 5.1



## 6 Preface to Section 6

Section 6 is a manuscript by Schwinghamer, Souleimanov, Dutilleul, and Smith that will be submitted to Biomass & Bioenergy. The literature cited in this section is listed in the reference section (page 160). Each figure caption, figure, and table is presented at the end of the section.

This section demonstrates the potential of acute treatments of  $10^{-6}$  M LCO, at planting and delivered subsequently as a foliar spray at the flowering stage of plant development, to modify the architecture and enhance factors that contribute to the seed weight and seed oil content of *B. napus* cultivars intended for agricultural use. This section also addresses the question of the effect due to an acute treatment of thuricin 17 at planting on factors that contribute to yield, under high temperature conditions.

The thesis consists of 4 papers: the first focused on the beginning of development, *i.e.*, germination; the second focused on subsequent growth and early development; and the third focused on development right through to yield, but using a rapid system. This is the fourth, which focuses on development, including plant architecture through to yield with conventional genotypes.

## **The response of canola (*Brassica napus* [L.]) agricultural cultivars to *Bradyrhizobium japonicum* 532C signal molecule lipo-chitooligosaccharide (Nod Bj V [C18:1, MeFuc]) and *Bacillus thuringiensis* endophytic bacterium 17 bacteriocin**

### **6.1 Abstract**

Lipo-chitooligosaccharides (LCOs) are signal molecules that play a role in initiating the symbiotic legume-rhizobia, *Parasponia*-rhizobia, and arbuscular mycorrhizal processes. Previous work has shown that the application of a  $10^{-6}$  M LCO solution, or a  $10^{-11}$  M thuricin 17 solution, can stimulate the germination and growth of a variety of non-leguminous plants. Therefore, the objectives were: to grow 04C111 under optimal temperature conditions in a greenhouse, and to assess the effect of LCO seed application and spray treatment on 04C111 floral initiation, architecture, and factors that contribute to yield; to assess the effect of a thuricin 17 seed application treatment upon factors that contribute to yield in a greenhouse system where 04C111 is grown under high temperature conditions; and to assess the effect of an LCO seed application and spray treatment on Topas floral initiation, architecture, and factors that contribute to yield, and seed quality, in a greenhouse system where Topas is grown under simulated cool spring conditions. *Brassica napus* cv. 04C111, grown from seed treated with LCO, produced  $0.6 \pm 0.2$  more primary branch than plants grown from untreated seed ( $p_{\text{diff}} = 0.0411$ ). Application of an LCO foliar spray increased the total of branch lengths per 04C111 plant by  $16.93 \pm 8.41$  cm ( $p_{\text{diff}} = 0.0425$ ). Based on the stem-to-branch length ratio, LCO-sprayed 04C111 plants were 68 % less apically dominant than unsprayed plants. Topas plants that were sprayed with LCO produced seed with  $\sim 4.45$  % more oil (% d.w.) than unsprayed plants. For both cultivars, seed treatment with a  $10^{-6}$  M concentration of LCO produced plants with a higher average sum of primary, secondary, and tertiary branch ( $\geq 1$  cm) frequencies.

### **6.2 Introduction**

The cultivated area of canola has been expanding rapidly in Canada, in part owing to the intensifying demand for biodiesel feedstock (Franzaring et al. 2008). Of the species of plants that are collectively referred to as canola, *Brassica napus* typically produces the highest yields, and 1 to 2 % more seed oil than *Brassica rapa* (Kirkland 1992). The

average yield of *B. napus* in Canada was 1.561 Mg ha<sup>-1</sup> in 2001 and 1.892 Mg ha<sup>-1</sup> from 2008 to 2010 (Statistics Canada 2012; Harker 2012b). Because of a very short growing season in Canada, particularly in the prairies, *Brassica napus* seed is usually planted in the early spring, when the temperature is below optimum, so that plants are able to reach maturity without any mid-summer heat-stress-related reduction in yield.

Natural plant growth regulators (PGRs) such as jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), and gibberellins (GAs) can influence the plant response toward abiotic stresses (Meng et al. 2009; Ullah 2012). The most economically important family of synthetic PGRs, the triazoles, are fungicides that regulate plant growth in part by inhibiting GA biosynthesis (Fletcher et al. 1986; Berry and Spink 2006). By blocking GA, triazoles reduce the height of *B. napus* and thereby diminish the risk of early lodging. By decreasing the incidence of lodging in *B. napus*, gibberellin-blocking PGRs increase the amount of harvestable seed and affect yield positively. However, triazoles persist in the soil long after use and they are neurotoxic to mammals (Daniels & Scarisbrick 1986; Crofton 1996). Other PGRs, such as auxin analogues or those that promote ethylene production, have also been used to retard *B. napus* growth and reduce lodging. The experiments reported here investigate two potential alternative PGRs. Previous experiments have shown that *B. napus* detects and responds to the bacterial products LCO and thuricin 17. Previously, a positive interaction was found on germination between *B. napus* cv. Topas and LCO under cool temperature conditions. Growth cabinet assays indicated that treatment with LCO can trigger higher emergence rates and produce multifoliate young plants, which could translate into increased and/or more rapid field surface coverage and crop dominance over weed populations at the critical early stage of growth. Under constant 30 °C temperature conditions, irrigation with thuricin 17 was found to reduce the lengths of petioles and reduce secondary embryogenesis, which was thought to be similar in effect to the GA-inhibition due to synthetic PGRs.

Lipo-chitooligosaccharide signals play a key role in mediating the early steps of legume and *Parasponia* infection by rhizobia and the resulting root nodule organogenesis, but they are not required in all instances (Giraud et al. 2007). The synthesis of the chitin

oligomer backbone of the LCO molecule requires the activity of three specific enzymes, encoded by the *nodABC* genes, which are present in all rhizobia characterized thus far and can be found in symbiotic plasmids or islands that may be mobilized by lateral transfer among rhizobia (Rogel et al. 2001, Sullivan and Ronson 1998). A single species of *Bradyrhizobium* can produce several unique LCOs, also called Nod factors (Carlson et al. 1993). Preliminary analysis suggested that the uncultured Dg1 symbiont of Durango root (*Datisca glomerata* [C. Presl] Baill.) contains *nodABC*-like genes, which suggests that it uses Nod factor-like compounds during the infection process, too (Persson et al. 2011). Arbuscular mycorrhizal (AM) fungi *Glomus intraradices* also secretes symbiotic sulphated and non-sulphated simple LCOs, which stimulate the formation of AM symbiosis in plant species of diverse families such as: *Fabaceae*, *Asteraceae*, and *Umbelliferae* (Maillet et al. 2011).

As a member of the Brassicaceae, *Brassica napus* does not have any specialized root adaptations for nutrient acquisition and has neither symbiotic nor mycorrhizal associations (Ocampo et al. 1980). However, plants that do not host rhizobia may nevertheless perceive LCO. Lipo-chitooligosaccharide molecules may be detected by brassicaceous plants via lysin motif (LysM)-containing chitin elicitor receptor kinases (RLKs) (Radutoiu et al. 2003). Five LysM RLK genes were identified in brassicaceous *Arabidopsis thaliana* [L.] Heynh., including RLK1/chitin elicitor receptor kinase 1 (CERK1), which induces plant defense after binding to chitin (Petutschnig et al. 2010). It binds to penta- to octameric chitin more strongly than chitin oligomers. Ohnuma et al. (2008) showed that the binding affinities of the LysM domains increase as the number of *N*-acetylglucosamine (GlcNAc) oligomer repeats increases.

The Nod factor applied was produced by *Bradyrhizobium japonicum*, Nod Bj V (C18:1, MeFuc) (MW 1415), which is a fatty acylated chitin pentasaccharide, *i.e.*, five GlcNAc residues with a 2-*O*-methyl-L-fucosyl residue attached to the 6-position of the reducing-end GlcNAc. Depending on the degree of saturation of the lipid chain, the relative orientation of the oligosaccharide and acyl moieties can range from being extended to essentially perpendicular to being quasi-parallel (Groves et al. 2005, Morando et al. 2011). It is likely that the lipid decoration of the LCO molecule is involved in the

specificity of perception (Gough and Cullimore 2011). Lipo-chitooligosaccharide is lipophilic. In aqueous solution, when the concentration is greater than mM, it forms large molecular weight aggregates, but it is likely monomeric at physiological concentrations (Groves et al. 2005). It is accumulated with relatively low levels of uptake, and concentrated and immobilized by binding sites in the plant cell wall, which become saturated by micromolar concentrations, before interaction with any receptors, such as LysM-RLKs in the plasma membrane (Goedhart et al. 2000). The lipophilic pathway through the cuticle involves solution and diffusion of the permeant within the lipophilic cuticular polymer, and this pathway could be particularly relevant with regards to the spray treatment used in these experiments (Schreiber 2005). The water-based LCO solution can be taken up without the use of a surfactant, by diffusion in water adsorbed to the stomatal pore walls (Eichert et al. 2008). In addition, microbial-associated molecular patterns may also enter plants via epidermal fissures generated by the emergence of lateral or adventitious roots, such as are typical of double haploid line *B. napus* varieties, and as have been reported by Sprent (2008) for the infection of tropical aquatic legumes of the *Aeschynomene* genus by *Bradyrhizobium* spp.

In *Arabidopsis* rosettes, treatment with  $10^{-6}$  M LCO solution can increase plant total ABA (10.19 %) and SA (15.00 %), and decrease indolylacetic acid (-49.68 %), cytokinin (-36.24 %), GA (-19.41 %), and JA (-33.66 %), 24 h post treatment (Subramanian, personal communication). Previously, salicylic acid treatment has been shown to improve *B. napus* cv. PF growth and increase the above ground dry weight (Cui et al. 2010; Kazemi et al. 2010).

*Bacillus thuringiensis* non-*Bradyrhizobium* endophytic bacterium 17 (BtNEB17) produces and excretes thuricin 17 (MW 3137.0). Thuricin 17 is a class IId bacteriocin with a broad range of antimicrobial activity against closely related bacterial species (Gray et al. 2006a). The peptide sequence was published by Lee et al. (2009). Although thuricin 17 has not been confirmed as a signal in the phloem, peptides such as thuricin 17 could generate or amplify messages, and act as signals themselves within the phloem of *B. napus* (Giavalisco et al. 2006). Class IId bacteriocins produced by *Bacillus* strains are highly effective inducers of plant defense-related enzymes phenylalanine ammonia lyase,

guaiacol peroxidase, ascorbate peroxidase, superoxide dismutase, and polyphenol oxidase (Jung et al. 2011). Previous work has shown that thuricin 17 can enhance soybean (*Glycine max* [L.] Merr.) and maize (*Zea mays* [L.]) growth (Lee et al. 2009). de Freitas et al. (1997) observed 30 to 54 % more siliquae produced by *B. napus* plants inoculated with a *B. thuringiensis* strain, which they attributed to the bacterial production of plant growth hormones.

The objectives were: to grow 04C111 under optimal temperature conditions in a greenhouse, and to assess the effect of LCO seed application and spray treatment on 04C111 floral initiation, architecture, and factors that contribute to yield; to assess the effect of a thuricin 17 seed application treatment upon factors that contribute to yield in a greenhouse system where 04C111 is grown under high temperature conditions; and to assess the effect of an LCO seed application and spray treatment on Topas floral initiation, architecture, and factors that contribute to yield, and seed quality, in a greenhouse system where Topas is grown under simulated cool spring conditions.

### **6.3 Materials and Methods**

Lipo-chitooligosaccharide and thuricin 17 were produced as described in section 3.3.2 and 3.3.3.

#### **6.3.1 Plant material**

For these experiments, a high erucic acid *B. napus* cultivar, 04C111(Peter McVetty, University of Manitoba), and Topas (SWAB, Svalöv, Sweden), a commercial-quality double-low cultivar, with low erucic acid and low glucosinolate seed protein, were used. The crosses that produced 04C111 are presented in standard breeder's notation, where '/' represents one cross, and increasing numbers of '/' marks indicates previous crosses, here: HiQ////Apollo///86LL141//Tatyoan/R83-14. The seed oil content of Topas is 42.2 % d.w and the seed oil content of 04C111 is 53.8 % d.w

#### **6.3.2 Conditions for plant growth**

The experimental cultivars were grown in separate greenhouse rooms, under different temperature conditions. The average daytime temperatures for the automatically regulated greenhouses are shown in Figure 6.1. Figure 6.2 shows the daily temperature

readings from an alcohol thermometer in a third, high temperature greenhouse. The experimental methodology was similar for all three greenhouse experiments. The growth medium was a 4 component mix 10:5:2:10 non-mycorrhizal promix: black earth: sand: vermiculite. The pots (25.4 cm diameter) were installed in McGill University's Plant Science Research Greenhouse, in Ste. Anne de Bellevue, Québec, on January 4 and 13, 2011, for the 04C111 and Topas experiments, respectively, which involved LCO treatments, and June 13, 2011, for the experiment involving thuricin 17. The greenhouses were illuminated at 800 to 1300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , for 16 h per day. In each pot, five seeds were planted on the surface of the growth medium. Seeds were treated with 10 mL of  $\mu\text{M}$  LCO solution, 10 mL  $10^{-11}$  M thuricin 17 solution, or 10 mL distilled water. Five mL of pelleted slow release fertilizer (Nutricote 14-14-14, [msds.plantprod.com/document/136](http://msds.plantprod.com/document/136)) was added to the original potting medium of each pot. Tepid tap water was provided as needed. At the third true leaf stage, each pot was thinned to 2 plants. When signs of phosphorous deficiency became evident, under cool spring conditions in the low temperature greenhouse, another 5 mL of fertilizer was added to the developing Topas plants.

Plants were either left unsprayed or foliar-sprayed with either 0.5 mL of distilled water or 0.5 mL of a  $\mu\text{M}$  LCO solution. Plants in the high temperature greenhouse experiment were not sprayed with LCO solution. Previous experiments had shown a positive interaction between treating the seed with signal solution and then spraying with water at the flowering stage. Hence, the plants in the high temperature greenhouse were seed-treated with thuricin 17 but sprayed only with water. Plants were removed from the greenhouse for spray treatment. The spray treatment was delivered to the upper sides of the leaves with an atomizer (Nalgene USA), at the flowering stage of plant development, *i.e.*, when daily counts of open flowers established that >75 % of plants bore open flowers, when the plants had achieved their maximum leaf area, which declines after anthesis (De Bock et al. 2012). Topas plants were sprayed 63 days after planting and 04C111 plants were sprayed 38 days after planting in the high temperature condition greenhouse and 62 days after planting in the optimal temperature condition greenhouse. Subsequently, plants were grown to senescence.

Plant growth and development was assessed by counting branches per plant and measuring the the height of the stem and the lengths of branches with a measuring tape. A distinction was made between those branches that arose from the main stem, namely ‘primary branches’ and those that arose from primary branches, namely ‘secondary branches.’ Open flowers were counted daily, until the plants were sprayed with their respective treatments. Late flowering from lateral branches, during the apical pod-filling stage of Topas plant development was quantified on April 8<sup>th</sup>, 2011.

Siliquae are pods with seed, whereas pods do not necessarily contain seed. The number of siliquae, seeds, and seeds per siliqua were counted. For the 04C111 experiments, seed weight was calculated on a per plant basis. For the Topas experiment, to guard against a position effect, a distinction was made between siliquae and seed borne on the apical inflorescence versus those borne by lateral branches (King et al. 1997). Oil, protein, and moisture percentages of *B. napus* cv. Topas apical seed dry matter were determined by NIR analysis (Deb Witko, Quality Laboratory, University of Manitoba).

### **6.3.3 Statistical analysis**

Average single seed weight and the ratios of apical:lateral siliquae and seed were calculated by SAS 9.3 (SAS Institute Inc., Cary, NC, USA) in the DATA step. SAS PROC UNIVARIATE was used to produce Shapiro-Wilk statistics to assess the distribution of the greenhouse data. Chi-squared tests were conducted using SAS PROC MIXED to assess the homogeneity of the variance. In the cases where the distribution of ANOVA residuals was not normal, outliers were identified based on Internally Studentized Residuals. Outliers were eliminated only if leverage statistics and Cook’s distance were extraordinary. In such cases, or where the pooling of plant data per pot did not normalize ANOVA residual distribution, nonparametric Kruskal-Wallis tests with adjustments for multiple comparisons were employed (Elliot and Hynan 2010). The number of observations (n), the mean ( $\mu$ ), and standard error (se) are reported. Results are reported at the  $p < 0.1$  level and p-values are indicated.

There were at most 7 primary branches per plant. Owing to the simple architecture of the 04C111 plants, the branch lengths were systematized. The 04C111 primary branch lengths were arranged from longest to shortest and numbered them from 1 to 7, *i.e.* the

most branched plant had 7 primary branches and 1 secondary branch. As such, the treatment effects were compared on the longest branches, second-longest branches, third-longest, *etc.* After ordering Topas primary branch data according to length, rather than position on the plant, it was detected that the primary branch length data were skewed to the right with high peaks. The variances were homogenous, but the residual frequencies weren't distributed normally. One plant consistently produced outlying branch lengths, but these outliers were not removable based on leverage statistics and Cook's distance. To normalize the residual frequency distribution, the Central Limit Theorem was employed. The plant data per pot was pooled. Measurements from apical or lateral Topas plant parts were considered separately. For example, in order to consider the apical dominance, which is one of the primary determinants of plant form, the apical ratio, *i.e.*, stem height to the sum of all primary, secondary, and tertiary branch lengths was calculated. Flowering on lateral branches was assessed once on Topas plants, on April 8, 2011, using SAS PROC GLIMMIX with binomial distribution (no flowers from lateral branches = 0, lateral branches flowering = 1).

For the analysis of correlations ( $\rho$ ) between the parameters, SAS PROC CORR was employed, and the SPEARMAN option was used to test the correspondence of paired data in the form of ranks. To produce a holistic picture of yield, SAS PROC IML was used to obtain path coefficients ( $r$ ). For the principal component analysis (PCA), the variables were ranked using PROC RANK in SAS 9.3 and then applied SAS PROC PRINCOMP (SAS Institute Inc., Cary, NC, USA). Some variables were removed from the PCA owing to their construction.

## **6.4 Results**

### **6.4.1 04C111 treated with LCO under optimal temperature conditions**

The Spearman correlation coefficients revealed positive associations between seed weight per plant (yield) and each of the predictor variables. Path analysis revealed there were interrelationships among the predictor variables. When these correlations were taken into consideration, the analysis indicated that seed number was strongly and positively associated with yield ( $p = 0.94347$ ,  $p < 0.0001$ ,  $r = 0.8244658$ ).

A Kruskal-Wallis test indicated that plants grown from seeds to which LCO had been applied produced approximately 1 more branch than the controls ( $n_0 = 29$ ,  $\mu = 4.1$  branches,  $se = 0.2$ ;  $n_{LCO} = 20$ ,  $\mu = 4.7$  branches,  $se = 0.2$ ;  $p_{0-LCO} = 0.0411$ ). Considering the  $p < 0.1$  level, plants grown from LCO-treated seed produced 2.4 cm longer fifth-longest primary branches, on average, than plants grown from untreated seed ( $n_0 = 29$ ,  $\mu = 4.5$  cm,  $se_0 = 1.4$ ;  $n_{LCO} = 20$ ,  $\mu = 6.9$  cm,  $se_{LCO} = 1.8$ ;  $p_{0-LCO} = 0.0811$ ). At the  $p < 0.1$  level, plants grown from LCO-treated seed also produced 0.9 cm longer sixth-longest primary branches, on average, than plants grown from untreated seed ( $n_0 = 29$ ,  $\mu = 0.7$  cm,  $se = 0.7$ ;  $n_{LCO} = 20$ ,  $\mu = 1.6$  cm,  $se = 0.9$ ;  $p_{0-LCO} = 0.0749$ ). A  $t$ -test indicated the LCO spray increased the total length of branches produced by 04C111 plants by 16.93 cm, on average ( $n_0 = 29$ ,  $\mu = 97.62$  cm,  $se = 8.01$ ;  $n_{LCO} = 20$ ,  $\mu = 114.55$  cm,  $se = 8.41$ ;  $p_{0-LCO} = 0.0425$ ) (Fig. 6.3a). Spray treatment with LCO also increased the lengths of the third-longest branches by 4.7 cm, on average ( $n_0 = 29$ ,  $\mu = 20.8$  cm,  $se = 2.1$ ;  $n_{LCO} = 20$ ,  $\mu = 25.5$  cm,  $se = 1.9$ ;  $p_{0-LCO} = 0.0895$ ). A  $t$ -test indicated that the LCO spray also increased the length of the fourth-longest branches by 5.32 cm, on average ( $n_0 = 29$ ,  $\mu = 11.93$  cm,  $se = 2.17$ ;  $n_{LCO} = 20$ ,  $\mu = 17.25$  cm,  $se = 2.09$ ;  $p_{0-LCO} = 0.0094$ ) (Fig. 6.3a). LCO spray also increased the lengths of the fifth-longest branches, by 3.5 cm on average, as compared to unsprayed plants ( $n_0 = 20$ ,  $\mu = 3.3$  cm,  $se = 1.2$ ;  $n_{LCO} = 20$ ,  $\mu = 6.8$  cm,  $se = 1.9$ ;  $p_{0-LCO} = 0.0892$ ). Based on the nonparametric comparison of stem-to-branch length ratios, unsprayed 04C111 plants were 21.9 % more apically dominant than water-sprayed 04C111 plants, at the  $\alpha = 0.1$  level ( $n_{H_2O} = 9$ ,  $\mu = 1.482$ ,  $se = 0.515$ ;  $n_0 = 20$ ,  $\mu = 1.806$ ,  $se = 0.481$ ;  $p_{0-H_2O} = 0.0811$ ). At the  $\alpha = 0.05$  level, unsprayed 04C111 plants were 68 % more apically dominant than LCO-sprayed 04C111 plants ( $n_0 = 20$ ,  $\mu = 1.806$ ,  $se = 0.481$ ;  $n_{LCO} = 20$ ,  $\mu = 1.075$ ,  $se = 0.067$ ;  $p = 0.0294$ ) (Fig. 6.3b).

The PCA indicated that, in this data set, for the multiple traits measured on experimental groups differing in the presence and absence of the LCO treatment, variability was associated with multiple sources. The first five PCs displayed eigenvalues greater than 1. Therefore, according to Kaiser's rule, the first five PCs were not trivial. The first PC accounted for 35.09 % of the variation, the second for 22.39 % of the variation, the third for 12.58 % of the variation, the fourth for 8.69 % of the variation, and the fifth for 6.71 % of the variation. The first PC was strongly positively correlated to the length of

the longest ( $\rho = 0.83233$ ,  $p < 0.0001$ ) and third-longest branches ( $\rho = 0.85481$ ,  $p < 0.0001$ ). The second PC was strongly positively correlated to the number of branches ( $\rho = 0.85783$ ,  $p < 0.0001$ ) and the length of the fifth-longest branch ( $\rho = 0.80985$ ,  $p < 0.0001$ ) and positively correlated to the presence of the LCO seed treatment ( $\rho = 0.44330$ ,  $p = 0.0047$ ). The third PC showed positive loading for the spray treatment, and weakly positive loading for the presence of LCO in the spray (Table 6.1). Subsequently, the third PC was labeled the “spray” component. The fifth PC showed strong positive loading for the LCO seed treatment, and it was labeled the “LCO” component. Thus, several key developmental aspects of plant development, such as branch length and number, are fundamental to the overall growth of the plant. Added to this is a clearly separate LCO effect.

#### **6.4.2 04C111 treated with thuricin 17 under high temperature conditions**

There were positive associations between yield and each of the predictor variables. Path analysis revealed there were interrelationships among the predictor variables. When these correlations were taken into consideration, the analysis indicated that, as under optimal temperature conditions, seed number was strongly and positively associated with yield, but the correlation was somewhat stronger under high temperature conditions ( $\rho = 0.96771$ ,  $p < 0.0001$ ,  $r = 0.852405$ ).

The thuricin 17 seed treatment aggravated sterility due to high temperature conditions, which resulted in reduced seed yield. A *t*-test indicated that the unsprayed 04C111 plants grown from untreated seed produced 7 more seeds, on average, than water-sprayed plants grown from thuricin 17-treated seed ( $n_{0+0} = 10$ ,  $\mu = 20.9$ ,  $se = 5.5$ ;  $n_{T17+H2O} = 10$ ,  $\mu = 13.8$ ,  $se = 4.7$ ;  $p_{(0+0)-(T17+H2O)} < 0.0001$ ) (Fig. 6.4). 04C111 plants grown from untreated seed produced 25.34 mg higher total seed weights than plants grown from thuricin 17-treated seed ( $n_0 = 20$ ,  $\mu = 53.04$  mg seeds,  $se = 13.71$ ;  $n_{H2O} = 20$ ,  $\mu = 27.70$  mg,  $se = 7.42$ ;  $p_{0-H2O} = 0.0579$ ).

The PCA indicated that, in this data set, for the traits measured from groups differing in the presence or absence of the thuricin 17 seed treatment, variability was associated with two major sources. The foremost source of variation was associated with the ability of

the plants to set seed, and that ability was certainly disabled under the high temperature greenhouse conditions. Only the first two components displayed eigenvalues greater than 1. Therefore, according to Kaiser's rule, the first two PCs were not trivial. The first PC accounted for 51.09 % of the variation, the second PC for 17.46% of the variation. The first PC showed positive loading for the numbers of siliquae, the numbers of seeds, and the seed weight plant<sup>-1</sup> (Table 6.2). The first PC was subsequently labeled the "yield" component. The yield component was strongly positively correlated to seed weight ( $\rho = 0.96666$ ,  $p < 0.0001$ ) and seed number ( $\rho = 0.95703$ ,  $p < 0.0001$ ) and the number of siliquae ( $\rho = 0.94787$ ,  $p < 0.0001$ ). The yield component was negatively correlated to the presence of the thuricin 17 seed treatment ( $\rho = -0.37481$ ,  $p = 0.0265$ ). The second PC showed strong positive loading for the spray treatment, and it was strongly positively correlated to the presence of the spray treatment ( $\rho = 0.86037$ ,  $p < 0.0001$ ). It was subsequently labeled the "spray" component. The spray component was positively correlated to the presence of the thuricin 17 seed treatment ( $\rho = 0.45431$ ,  $p = 0.0061$ ). Therefore, the components of plant fertility are fundamental, and there was a clearly separate effect owing to the spray treatment, indicating regulatory effects of these treatments on the overall growth and development of the plants.

#### **6.4.3 Topas treated with LCO under simulated cool spring conditions**

There were strong positive associations between the number of apical pods and the number of apical seeds with seed oil content,  $\rho = 0.90109$  and  $0.93722$ , respectively ( $p < 0.0001$ ). Path analysis revealed there were interrelationships among the predictor variables. When these correlations were taken into consideration, the analysis indicated that the number of apical seeds was positively associated with seed oil content ( $r = 0.730119$ ).

Topas flowering began on March 15, 2011, five days before the equinox. On day 61 after planting (DAP), the plants grown from untreated seed had approximately 1 more open flower, than the plants grown from LCO-treated seed ( $n_0 = 30$   $\mu = 1.8$  flowers,  $se = 0.4$ ;  $n_{LCO} = 19$ ,  $\mu = 0.8$  flowers,  $se = 0.4$ ;  $p_{0-LCO} = 0.0284$ ) (Fig. 6.5). On 62 DAP, if we consider the  $p < 0.1$  level, the day prior to spraying, plants that were grown from untreated seed had approximately 3 more open flowers than plants grown from LCO-

treated seed ( $n_0 = 30$ ,  $\mu = 7.1$  flowers,  $se = 0.9$ ;  $n_{LCO} = 19$ ,  $\mu = 4.3$  flowers,  $se = 0.9$ ;  $p_{0-LCO} = 0.0673$ ). Considering the  $p < 0.1$  level, an assessment of whether or not lateral branches were flowering on April 8, 2011, revealed that the LCO spray inhibited late flowering from Topas lateral branches by 38.7 % ( $n_0 = 19$ ,  $\mu = 73.7$  %,  $se = 10.3$ ;  $n_{H_2O} = 10$ ,  $\mu = 70.0$  %,  $se = 15.3$ ;  $n_{LCO} = 20$ ,  $\mu = 35.0$  %,  $se = 10.9$ ;  $p_{0-LCO} = 0.0764$ ) (Fig. 6.6a).

Considering the  $p < 0.1$  level, approximately 3 more branches  $\geq 1$  cm (primary, secondary, and tertiary branches) were produced by Topas plants grown from LCO-treated seed than from plants grown from untreated seed ( $n_0 = 30$ ,  $\mu = 8.5$  branches,  $se = 0.9$ ;  $n_{LCO} = 18$ ,  $\mu = 11.9$  branches,  $se = 2.0$ ;  $p_{0-LCO} = 0.0751$ ). On average, approximately 3 more secondary branches were produced by plants grown from LCO-treated seed than from those plants grown from untreated seed ( $n_0 = 30$ ,  $\mu = 3.1$  branches,  $se = 0.7$ ;  $n_{LCO} = 18$ ,  $\mu = 5.7$  branches,  $se = 1.7$ ;  $p_{0-LCO} = 0.0973$ ). However, unsprayed plants produced approximately 3 more secondary branches than LCO-sprayed plants ( $n_0 = 18$ ,  $\mu = 11.5$  branches,  $se = 1.7$ ;  $n_{LCO} = 20$ ,  $\mu = 8.65$  branches,  $se = 1.5$ ;  $p_{0-LCO} = 0.0972$ ). LCO-sprayed Topas plants had sums of primary branch lengths that were 64.6 cm shorter, on average, than those plants that were left unsprayed ( $n_0 = 18$ ,  $\mu = 244.8$  cm,  $se = 40.2$ ;  $n_{LCO} = 20$ ,  $\mu = 180.2$ ,  $se = 23.7$ ;  $p_{0-LCO} = 0.0737$ ). A  $t$ -test indicated that the longest primary branches from unsprayed plants were 13.43 cm longer on average than the longest branches produced by LCO-sprayed plants ( $n_0 = 18$ ,  $\mu = 56.31$  cm,  $se = 6.24$ ;  $n_{LCO} = 20$ ;  $\mu = 42.88$  cm,  $se = 3.14$ ;  $p_{0-LCO} = 0.0394$ ) (Fig. 6.6b). At the  $p < 0.1$  level, the second-longest branches from unsprayed plants were 13.4 cm longer than the second-longest branches produced by LCO-sprayed plants ( $n_0 = 18$ ,  $\mu = 49.5$  cm,  $se = 5.9$ ;  $n_{LCO} = 20$ ;  $\mu = 39.3$  cm,  $se = 2.9$ ;  $p_{0-LCO} = 0.0520$ ). Similarly, the third-longest primary branches on unsprayed plants were 9.6 cm longer than those produced by LCO-sprayed plants ( $n_0 = 18$ ,  $\mu = 43.1$  cm,  $se = 5.6$ ;  $n_{LCO} = 20$ ;  $\mu = 33.5$  cm,  $se = 3.5$ ;  $p_{0-LCO} = 0.0695$ ). And the fourth-longest primary branches from unsprayed plants were 10.9 cm longer than the second-longest branches produced by LCO-sprayed plants ( $n_0 = 18$ ,  $\mu = 36.7$  cm,  $se = 5.8$ ;  $n_{LCO} = 20$ ;  $\mu = 25.8$  cm,  $se = 3.9$ ;  $p_{0-LCO} = 0.0705$ ).

A  $t$ -test indicated that the apical inflorescences of Topas plants sprayed with LCO produced, on average, 9.1 more siliquae than unsprayed plants ( $n_0 = 16$ ,  $\mu = 24.4$ ,  $se =$

3.3;  $n_{\text{LCO}} = 20$ ,  $\mu = 33.5$ ,  $se = 2.5$ ;  $p_{0-\text{LCO}} = 0.0193$ ) (Fig. 6.6c). Correspondingly, Topas plants sprayed with LCO produced, on average, 0.1 more apical siliquae per cm of stem ( $n_0 = 16$ ,  $\mu = 0.2 \text{ cm}^{-1}$ ,  $se = 0.0$ ;  $n_{\text{LCO}} = 20$ ,  $\mu = 0.3 \text{ cm}^{-1}$ ,  $se = 0.0$ ;  $p_{0-\text{LCO}} = 0.0313$ ). A nonparametric test indicated that the Topas plants that were sprayed with LCO had higher ratios of apical:lateral siliquae – 76.1 % higher, on average - than unsprayed plants ( $n_0 = 16$ ,  $\mu = 0.406$ ,  $se = 0.067$ ;  $n_{\text{LCO}} = 20$ ,  $\mu = 0.715$ ,  $se = 0.109$ ;  $p_{0-\text{LCO}} = 0.0238$ ) (Fig. 6.6d).

When the  $p < 0.1$  level was considered, the water-sprayed plants produced approximately 30 more seeds, on average, than the unsprayed plants ( $n_{\text{H}_2\text{O}} = 10$ ,  $\mu = 353.6$  seeds,  $se = 31.5$ ;  $n_0 = 16$ ,  $\mu = 323.4$  seeds,  $se = 58.9$ ;  $p_{0-\text{H}_2\text{O}} = 0.0856$ ). Topas plants grown from untreated seed produced 374.31 mg higher seed weights at harvest, from their apical inflorescences, than those grown from LCO-treated seed ( $n_0 = 29$ ,  $\mu = 2.01851$  g,  $se = 0.13263$ ;  $n_{\text{LCO}} = 17$ ,  $\mu = 1.64421$ ,  $se = 0.18859$ ;  $p_{0-\text{LCO}} = 0.0712$ ), and LCO-sprayed Topas apical inflorescences produced 0.42 mg lower mean single seed weights than unsprayed plants ( $n_0 = 16$ ,  $\mu = 5.67$  mg,  $se = 0.35$ ;  $n_{\text{LCO}} = 20$ ,  $\mu = 5.25$  mg,  $se = 0.50$ ;  $p_{0-\text{LCO}} = 0.0978$ ). However, Topas plants that were seed-and-spray-treated with LCO produced higher oil (4.45 % d. w.) seed than those grown from untreated seed and sprayed with water ( $n_0 = 6$  plants,  $\mu = 47.09564660$  %,  $se = 0.78962492$ ;  $n_{\text{LCO}} = 3$  plants,  $\mu = 51.54635240$  %,  $se = 0.23366385$ ;  $p_{0-\text{LCO}} = 0.0635$ ).

The PCA indicated that the LCO spray was positively correlated with a major source of variability that affected the components of yield. As such, the spray seemed to produce more successful pollination, under the conditions of this system. According to Kaiser's rule, the first five PCs were not trivial. The first PC accounted for 40.72 % of the variation, the second PC for 22.42 % of the variation, the third for 9.31 % of the variation, the fourth for 6.95 % of the variation, and the fifth for 5.75 % of the variation. The eigenvectors for the first five PCs are shown in (Table 6.3). The first PC was strongly positively correlated to the height of the stem ( $\rho = 0.90507$ ,  $p < 0.0001$ ) and the lengths of the longest ( $\rho = 0.90838$ ,  $p < 0.0001$ ), second-longest ( $\rho = 0.94210$ ,  $p < 0.0001$ ), third-longest ( $\rho = 0.95421$ ,  $p < 0.0001$ ), fourth-longest ( $\rho = 0.92609$ ,  $p < 0.0001$ ), and fifth-longest branches ( $\rho = 0.88736$ ,  $p < 0.0001$ ). The second PC was strongly positively correlated with the number of apical seeds ( $\rho = 0.92208$ ,  $p < 0.0001$ ), the number of

apical siliquae ( $\rho = 0.87492$ ,  $p < 0.0001$ ), and the apical seed weight ( $\rho = 0.84545$ ,  $p < 0.0001$ ). The second PC was also positively correlated to the LCO spray treatment ( $\rho = 0.65153$ ,  $p = 0.0014$ ). Thus, several factors that contribute to yield were not separate from the effect of the LCO spray. The fifth PC was positively loaded for the spray treatment, and it was subsequently labeled “spray” (Table 6.3).

#### **6.4.4 Results from the correlation analysis**

The stronger correlations, produced by the LCO seed treatment, indicated that LCO-treated plants with shorter branches produced more seed from the apical inflorescence. For the plants produced from untreated seed, the distribution of siliquae was less strongly correlated to plant architecture. That is, the 95 % confidence interval for the Spearman correlation statistic for the sums of all branch lengths with the ratios of apical:lateral siliquae produced by Topas plants grown from untreated seed ( $\rho = -0.756671$  to  $-0.400084$ ,  $p = 0.0003$ ) did not overlap with that of plants grown from LCO-treated seed ( $\rho = -0.977849$  to  $-0.900805$ ,  $p < .0001$ ). Similarly, the 85% confidence interval for the Spearman correlation statistic for the ratios of stem heights:sums of all branch lengths with the ratios of apical:lateral siliquae produced by Topas plants grown from untreated seed ( $\rho = 0.465716$  to  $0.789166$ ,  $p < .0001$ ) did not overlap with that of plants grown from LCO-treated seed ( $\rho = 0.818797$  to  $0.958133$ ,  $p < .0001$ ). Among the unsprayed Topas plants, those that produced fewer branches, and fewer and shorter secondary branches, also produced relatively more siliquae on the apical inflorescence, compared to plants sprayed with water or LCO, for which the negative correlation wasn't as strong. The positive correlation between secondary branch number and length was strong for all of the experimental plants, but it was stronger for LCO-sprayed plants (Table 6.4).

### **6.5 Discussion**

Many of the young 04C111 plants produced 3 or 4 cotyledons, regardless of whether or not they were treated with LCO. Polycotyledony is a type of altered phyllotaxy. Altered phyllotaxy, adventitious roots, and vascular differentiation, such as among the 04C111 plants with multiple stems, are all regulated by auxin. These characteristics were typical of this cultivar, which indicated a high level of auxin, or sensitivity to auxin, in 04C111.

Lipo-chitoooligosaccharides have been reported to have mitogenic and morphogenic effects similar to cytokinins and inhibitors of auxin transport (Hirsch et al. 1989; Hirsch and Fang 1994 and references therein) and Subramanian (2014) observed a dramatic decrease in indolylacetic acid in LCO-treated *Arabidopsis* rosettes. It may also interest future researchers that, among the Topas plants, floral reversion was observed, regardless of treatment (Madlung et al. 2011).

The findings for 04C111 are different from the findings for Topas, and the experimental cultivars had different responses to LCO application, but plants treated with LCO produced more branches (1 for 04C111 and 3 for Topas), and were therefore relatively ramose, compared to plants grown from untreated seed. Branching patterns are normally determined by light, nutrients, and genotype. Stand density and applied nitrogen are well known factors that influence the total numbers of branches and the numbers of secondary branches produced by *B. napus* (e.g., Jixian and Hua 1997). Physiologically, branching is regulated by hormones including cytokinin and auxin. Cytokinin stimulates cell division activity and outgrowth when applied directly to the axillary buds of many species. The initiation and normal development of floral buds of *B. napus*, *in vitro*, from sepal primordia stage to maturity, is dependent on the presence of cytokinin, and is sensitive to the initial pH of the growth medium (Polowick & Sawhney 1991). Benzylaminopurine was more effective than other cytokinins (kinetin, zeatin, and zeatin riboside) for the *in vitro* stimulation of the initiation of additional buds on the *B. napus* inflorescence axis. Auxin transported from the apical bud inhibits the expression of a subset of genes that encode the isopentenyltransferase enzyme that catalyzes the first committed step to cytokinin biosynthesis. It has been noted previously in *Brassica* species that lowering the auxin:cytokinin ratio increases the number of primordia that develop into lateral buds, e.g., exogenous cytokinin treatment has stimulated bud outgrowth. Plants can repress auxin signaling as a component of basal resistance and alkalinization such as typically precedes defense responses, and such as has been implicated in signal chains mediating defense response induction that can be triggered by LCO (Staehelin et al. 1994). Plant growth regulators in the triazole group are known to increase cytokinin levels. By inhibiting gibberellin and sterol synthesis, increasing water content, reducing water consumption, and increasing the CO<sub>2</sub> fixation rate, triapenthenol (RSW0411) treatment at

the bud stage can reduce *B. napus* height and increase branching, total pods plant<sup>-1</sup>, and yield (Daniels & Scarisbrick 1986; Kikland 1992). As such, the LCO spray had a comparable effect on Topas, under simulated cool spring conditions. Plant growth regulators that are growth retardants in the triazole family are applied widely in *B. napus* agriculture, but are known to be neurotoxic to mammals and persistent in the soil long after use (Daniels & Scarisbrick 1986; Crofton 1996).

In *G. max*, the putative products of genes down regulated by LCO include a number of proteins related to auxin, such as AUX1-like auxin carrier protein (Timppte et al. 1995) and auxin-binding protein ABP20 precursor (Wang et al. 2012). Similarly, the NAC (NAM, ATAF1.2, CUC2) transcription factors, which are represented by a large gene family diverse in structure and function. The NAC family have been shown to regulate, among other things, auxin signaling (Hegedus et al. 2003; Nakashima et al. 2007). *Glycine max* antioxidant genes were responsive to Wang et al.'s (2012) LCO foliar spray, notably the up-regulated glutathione S-transferase, which is involved in auxin and cytokinin metabolism. Plant peroxidases (POXs) are bifunctional enzymes involved in both the reduction of H<sub>2</sub>O<sub>2</sub> and the production of reactive oxygen species during oxidative burst (Bae et al. 2006). POXs also participate in auxin catabolism, as well as light-mediated developmental responses, *etc.* (Bae et al. 2006; Passardi et al. 2004). Dyachok (2002) compared LCO's action to that of cytokinin. Wang et al. (2012) found glutamate carboxypeptidase II to be up regulated in LCO-treated *G. max*. Helliwell et al. (2001) and Vidaurre et al. (2007) noted that glutamate carboxypeptidase II is involved in the development of shoot apical meristems, flowering, photomorphogenesis and cytokinin biosynthesis. Furthermore, with respect to the increased branching observed among LCO treated plants, such a response could also be a product of changes to the plant response to light. Wang et al. (2012) found LCO spray treatment suppressed two copies of BTB/POZ protein in *G. max*. The BTB (Broad Complex, tramtrack and bric a brac) or POZ (poxvirus and zinc finger) is a conserved domain found at the NH<sub>2</sub>-terminal of zinc fingers, poxvirus, and actin-binding proteins; it is a protein-protein interaction motif involved in the organization of micromolecular complexes (Albalgli et al. 1995). These were homologous to the phototropic-response protein NPH3 (nonphototropic

hypocotyl3) which is a UV-A/blue light receptor inducing the phototropic response (Sakai et al. 2000).

It would seem that LCO application might be useful in the case of thin stands, to encourage more branching and more potential seed production per plant. The production of more seed-yielding branches, such as was observed among LCO-treated plants (1 for 04C111 and 3 for Topas), may enable those plants to compensate for environmental stress and herbivory, such as pollen beetle attacks (Thomas 2003; Médiène et al. 2011). In this case, and in cases where the crop is decapitated or damaged, the LCO spray treatment could be used to encourage productivity. Branch number has been identified by other groups as an agronomic trait that affects indirectly the yield of winter and spring *B. napus* varieties, by affecting other yield components in negative or positive ways (Diepenbrock 2000; Aytac et al. 2008).

Although the greenhouse may have been lacking in pollinator insects, high rates of flowering, pollination, and seed set was observed. Therefore, it may be assumed that the high air exchange and turbulence in the greenhouse caused sufficient pollination. In any case, *B. napus* is self-pollinating and does not need wind and insects in order to set seeds. Although attractive to bees (*Apis mellifera* [L.]), 80 % of the seed produced originates from self-pollination (Franzaring et al. 2011). The sterility and consequently low yields observed among the thuricin 17-treated plants can be explained in part by the negative effect of the high temperature environment on pollen tube formation. High temperature stress, such as 22 or 25.5 °C daily mean temperature conditions, or daily temperature elevations from 21 to 24 °C during anthesis, produces pollen with reduced viability and germinability, and thin pollen tubes that have stunted and convoluted morphology, which reduce seed development, yield, and oil production (Nuttall et al. 1992; Young et al. 2004). After pollination, heat can induce leaf shedding and hasten maturity, which can result in a source limitation for seed yield (Gan et al., 2004). However, trends in reductions to total seed number and seed mass per pod, owing to elevated temperature, are specific to particular cultivars, e.g., Bolero and Mary yields are decreased under elevated temperature conditions (Frenck et al. 2011). The response of *B. napus* to signal molecules produced by *B. thuringiensis* is likely context-dependent. Similar to the

response to thuricin 17 under high temperature conditions, the application of the synthetic PGR cerone (ethephon), which generates ethylene, can cause abortion of entire racemes, particularly if applied at the green bud stage (Daniels & Scarisbrick 1986). There are still many things to learn about the response of *B. napus* to the thuricin 17 signal. Although the application of thuricin 17 can aggravate sterility under heat stress conditions, previous experiments indicated that positive results may be forthcoming under cold and saline conditions, both of which can be challenges to canola production in parts of Western Canada.

The 04C111 plants in the optimal temperature greenhouse and the Topas plants in the simulated cool spring greenhouse both started to flower as the Spring Equinox approached and the days became longer, as could be expected of this long day species. Increased transpiration, owing to stomatal conductance or membrane permeability (unpublished data), contributed to the lower leaf temperatures seen in Topas plants due to the LCO treatment. The ramose form of LCO-treated plants likely contributed to the regulation of leaf temperature, as it was measured from relatively basal leaves, which were relatively protected and shaded. The lower temperature of LCO-treated Topas plants likely contributed to the slower floral initiation. Although LCO-treated Topas plants flowered later, the proliferation of secondary branches and the continued formation of side shoots carrying new flowers resulted in larger vegetative structures and extended flowering, which produced more siliques and increased yields (Mendham and Salisbury 1995, Franzaring et al. 2011). Flowering delays in an agricultural setting can increase the risk of convoluted pollen tube morphology due to late summer high temperatures (Kutcher et al. 2010). Early crop maturity increases the likelihood of a successful harvest with high quality seed, because a relatively early harvest reduces the potential for green seed caused by frost, which decreases the grade and price. Recently, Takashima et al. (2013) found that the occurrence of frost during flowering was the main limiting factor to *B. napus* yields, in the southeastern Pampas of Argentina. Some synthetic PGRs delay flowering, notably paclobutrazol (PP333), a triazole that blocks the enzyme kaurene oxidase that catalyzes the oxidation of *ent*-kaurene to *ent*-kaurenoic acid, thereby inhibiting GA synthesis (Rademacher et al. 1984). High doses of PP333 can delay or

inhibit flowering (Rood et al. 1987, 1989). Applied as a root drench or foliar spray, paclobutrazol delayed the flowering of fully vernalized winter cv. Crystal (Zanewich 1993). Similarly, intermediate doses of chlormequat delayed flower development (Rood et al. 1987).

The combined seed-and-spray treatment increased seed oil by approximately 4.45 % on average, and decreased seed protein content, by approximately 3 % on average, as compared to the seeds produced by water-sprayed plants grown from untreated seed ( $n_{0+H_2O} = 6$  plants,  $\mu = 21.25259630$  %,  $se = 0.71149460$ ;  $n_{LCO+LCO} = 3$  plants,  $\mu = 18.45614430$  %,  $se = 0.33048025$ ;  $p = 0.0593$ ). This seems promising, considering the inverse relationship of oil and protein content that is characteristic to *B. napus* seed. As numerous groups have found, any condition that improves yield may potentially reduce the concentration of seed protein (Triboi and Triboi-Blondel 2002). The 4.45 % higher oil content from LCO-sprayed Topas plants promises higher seed oil yields, if the effect can be reproduced in an agricultural system. Similarly, the foliar application of  $10^{-5}$  M SA can, after the imposition of drought stress, overcome the drought-induced decrease of seed oil oleic acid for cvs. Rainbow and Dunkeld (Ullah et al. 2012).

## 6.6 Conclusions

Greenhouse experiments showed that LCO-seed-treated 04C111 plants grown to the harvest stage generate approximately 1 more branch, on average, as compared to plants grown from untreated seed. The total branch length of LCO-sprayed 04C111 plants was 16.93 cm longer, and therefore apical dominance was decreased by 17.94 % (Figs. 6.3a and b). The effect of thuricin 17 seed application seems to be relatively disadvantageous: unsprayed 04C111 plants grown from untreated seed in a high temperature greenhouse produced 7 more seeds, on average, than water-sprayed plants grown from seed treated by 10 mL  $10^{-11}$  M thuricin 17 (Fig. 6.4). The application of LCO to Topas seed, which was germinated and grown for 2 months under 10 °C conditions in a greenhouse before step-wise temperature elevation, delayed flowering slightly but significantly from the apex (Figure 6.5). The longest primary branches grown by unsprayed Topas plants were 13.43 cm longer on average than the longest branches produced by LCO-sprayed plants (Fig 6.6b). Topas plants that were sprayed with LCO produced approximately 9 more

siliquae, and 76.1% higher ratios of apical:lateral siliquae, than unsprayed plants (Fig 6.6c and d).

## 6.7 Tables

**Table 6.1 Eigenvectors for 04C111 plants grown under optimal temperature conditions in a greenhouse**

	PC1	PC2	Spray	PC4	LCO
Presence of LCO seed treatment	0.01824	0.23176	-0.0811	0.3044	0.76456
Presence of LCO in spray	0.12988	0.10706	0.49857	-0.438	0.25713
Presence of spray	0.21123	0.04314	0.51706	-0.3516	-0.0394
Primary stem height	0.33066	-0.0972	0.07563	0.32372	-0.2368
Secondary stem height	-0.1433	0.33638	-0.1191	-0.0489	-0.4158
Number of branches	0.09993	0.47048	0.17283	0.22329	-0.0571
Longest branch length	0.36226	-0.2449	-0.0045	0.04272	0.11166
Second-longest branch length	0.35215	-0.2347	-0.1107	0.13784	0.05084
Third-longest branch length	0.38156	-0.1593	-0.0381	0.08055	0.09736
Fourth-longest branch length	0.31573	0.09296	0.26298	0.25075	-0.1772
Fifth-longest branch length	0.11604	0.39595	0.19771	0.33782	-0.1102
Sixth-longest branch length	-0.0158	0.42389	-0.1294	-0.0532	0.17398
Number of siliquae	0.32563	0.15935	-0.3077	-0.0938	-0.1175
Number of seeds	0.28014	0.19497	-0.3321	-0.3689	0.04337
Seed weight	0.31558	0.20012	-0.29	-0.2878	-0.0395

**Table 6.2 Eigenvectors for 04C111 plants grown under high temperature conditions in a greenhouse**

	Yield	Spray
Presence of thuricin 17 seed treatment	-0.2164	0.37233
Presence of water spray treatment	-0.0317	0.89071
Number of pods	0.21507	-0.2045
Number of siliquae	0.54652	0.09905
Number of seeds	0.54906	0.10222
Seed weight	0.55296	0.07699

**Table 6.3 Eigenvectors for Topas plants grown under simulated cool spring conditions in a greenhouse**

	PC1	PC2	PC3	Spray	PC5
Length of siliqua	-0.1893	0.07689	0.16385	0.02014	-0.017
Seed protein content	0.17125	-0.3149	-0.1027	0.16343	0.15928
Seed oil content	-0.174	0.31805	0.04179	-0.193	-0.1514
Seed moisture content	-0.0803	-0.1226	0.19047	0.07199	0.445
Number of apical siliquae	-0.0518	0.37418	0.1576	0.16727	-0.2228
Number of lateral siliquae	0.21816	0.1893	0.30981	-0.1037	-0.0745
Number of apical seeds	-0.1128	0.38097	0.15055	-0.0125	-0.0661
Apical seed weight	0.04474	0.36792	0.1875	0.18933	-0.123
Number of primary branches	0.21826	0.1717	0.03429	-0.3699	0.26954
Number of secondary branches	0.28524	-0.0127	-0.1557	0.11139	-0.0842
Stem height	0.30395	-0.0587	0.11483	0.04572	-0.1228
Longest branch length	0.30214	-0.0469	0.12337	0.05049	-0.0911
Second-longest branch length	0.30844	-0.0707	0.11901	0.09411	-0.0277
Third-longest branch length	0.30845	-0.0262	0.14853	0.07468	-0.0941
Fourth-longest branch length	0.28958	0.01042	0.22907	-0.1211	0.01443
Fifth-longest branch length	0.27754	0.05297	0.26432	-0.029	0.12984
Sixth-longest branch length	0.19325	0.12956	0.01718	-0.3405	0.33985
Seventh-longest branch length	0.19277	0.217	-0.4194	-0.0092	-0.0057
Eighth-longest branch length	0.19277	0.217	-0.4194	-0.0092	-0.0057
Ninth-longest branch length	0.19277	0.217	-0.4194	-0.0092	-0.0057
Presence of LCO seed treatment	-0.1285	0.10072	0.01817	-0.3276	0.37226
Presence of spray treatment	0.04043	0.14155	0.04761	0.59453	0.35297
Presence of LCO spray treatment	-0.0479	0.3005	-0.0533	0.30817	0.41248

<b>Table 6.4 Spearman correlation coefficients for Topas plant architectural variables (LCO-sprayed/water-sprayed/unsprayed)</b>						
	Sum of all branch lengths	Apical pod number : lateral pod number	Height : branch length	Sum of secondary branch lengths	Number of secondary branches	Number of branches of all types
Sum of all branch lengths	1					
Apical pod number : lateral pod number	-0.7123*	1				
Height : branch length	-0.9402**	0.62707*	1			
Sum of secondary branch lengths	0.697*	-0.40735‡	-0.61611*	1		
Number of secondary branches	0.70446*	-0.41793‡	-0.60056*	0.99521***†	1	
Number of branches of all types	0.82894**	-0.50554*‡	-0.74121*	0.89237**	0.89494***†	1

\*  $p < 0.05$

\*\*  $p < 0.0001$

† indicates 90 % confidence interval does not overlap with that of unsprayed plants

‡ indicates 95 % confidence interval does not overlap with that of unsprayed plants

## 6.8 Figure captions

**Figure 6.1** Average daytime greenhouse temperatures for two greenhouses

**Figure 6.2** Daily temperature readings from the high temperature greenhouse

**Figure 6.3** (A) Sums of branch lengths of 04C111 plants and lengths of fourth-longest branches. (B) Apical dominance expressed as stem height (cm) / sum of branch lengths (cm). Bars indicate standard error, letters indicate difference ( $p < 0.05$ ).

**Figure 6.4** Mean per pot of seed produced per plant. Treatment 1 = control; 2 = seed-treated with 10 mL  $10^{-11}$  M thuricin 17; 3 = sprayed with 1.25 distilled water mL per plant at the flowering stage; 4 = seed + spray treatment. Bars indicate standard error, lower case letters indicate difference ( $p < 0.05$ ).

**Figure 6.5** Scatterplot smoothing (smooth = 0.5,  $\alpha = 0.05$ ) illustrating the numbers of open flowers on *B. napus* cv. Topas plants. A = seed treated with 10 mL 1  $\mu$ M LCO. B = control. The vertical line indicates the application of the foliar spray treatment.

**Figure 6.6** (A) *B. napus* cv. Topas plants with open flowers on lateral branches, April 8, 2011, sprayed at the flowering stage with  $\mu$ M LCO. (B) Differences in Topas primary branch lengths owing to LCO spray. (C) Siliquae produced on the apical raceme by *B. napus* cv. Topas plants grown under cool spring conditions. (D) Ratios of apical:lateral siliquae from *B. napus* cv. Topas, grown under cool spring conditions. Bars indicate standard error, letters indicate difference ( $p < 0.05$ ).

## 6.9 Figures

Figure 6.1

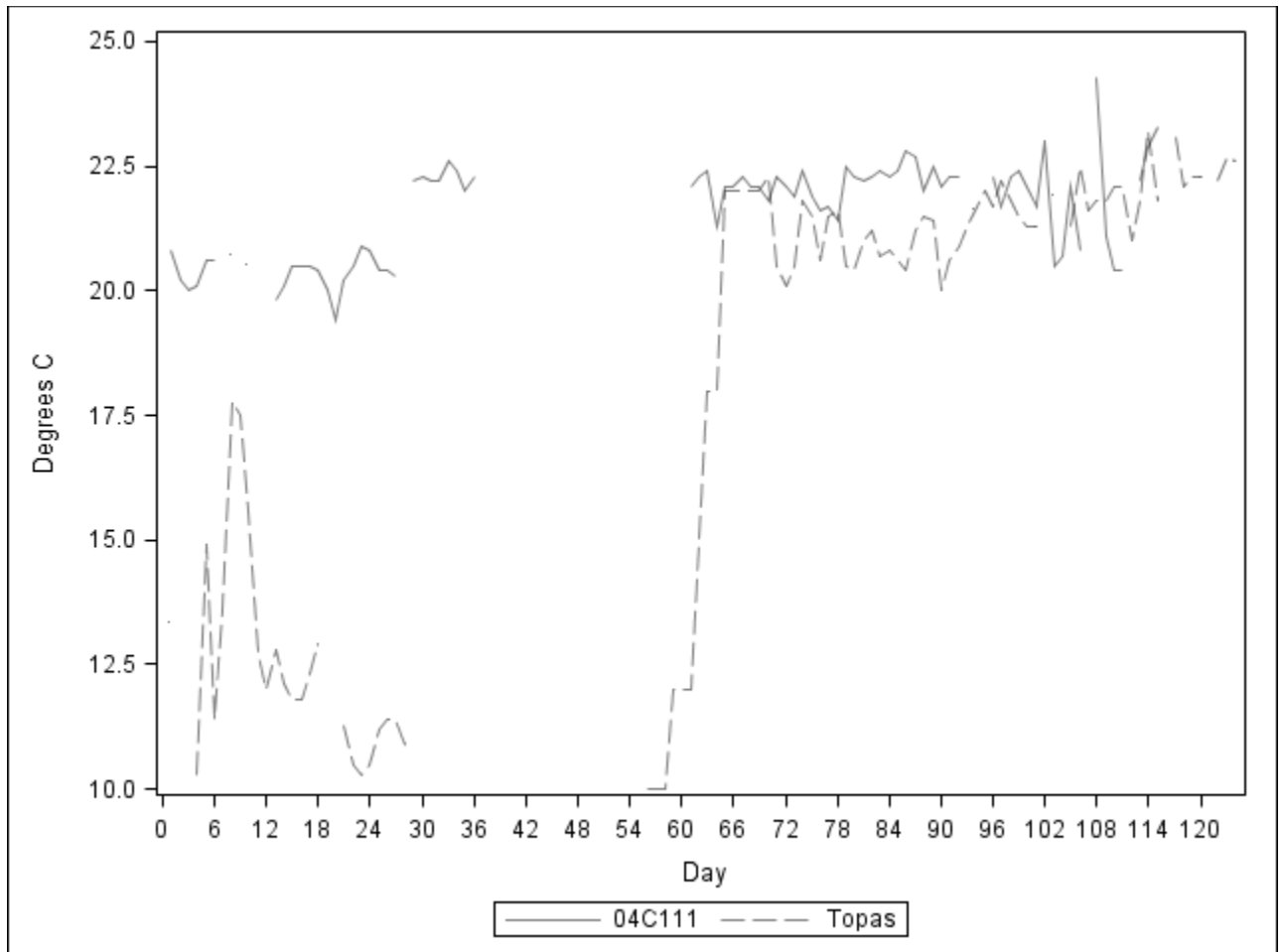


Figure 6.2

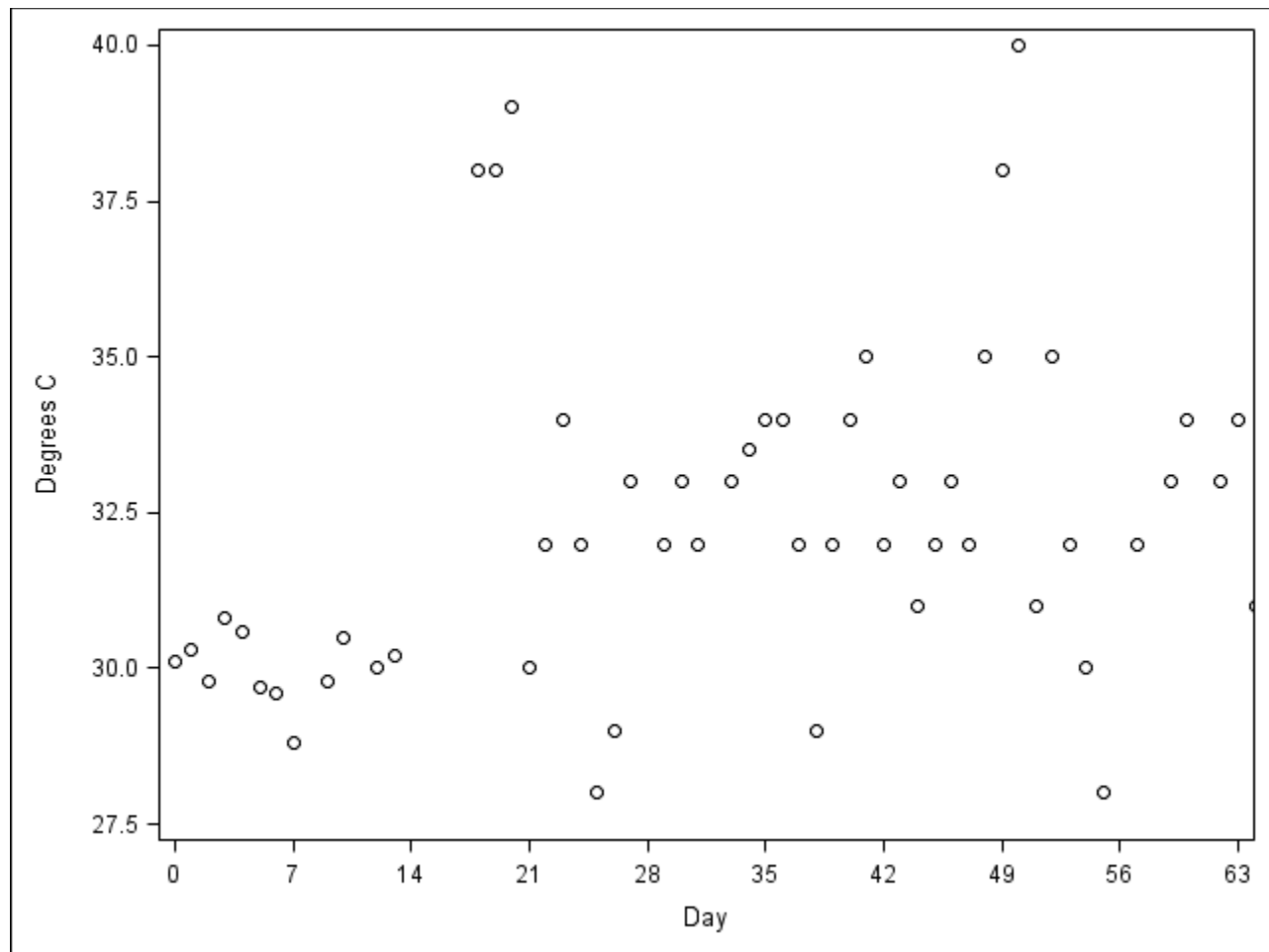
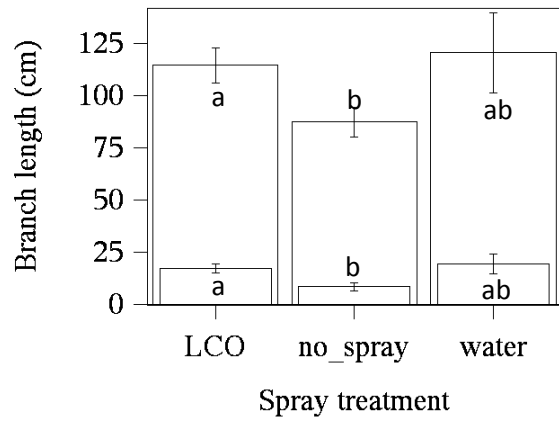


Figure 6.3

A)



B)

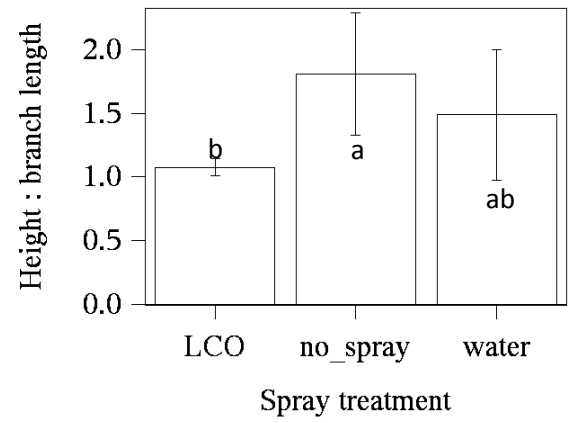


Figure 6.4

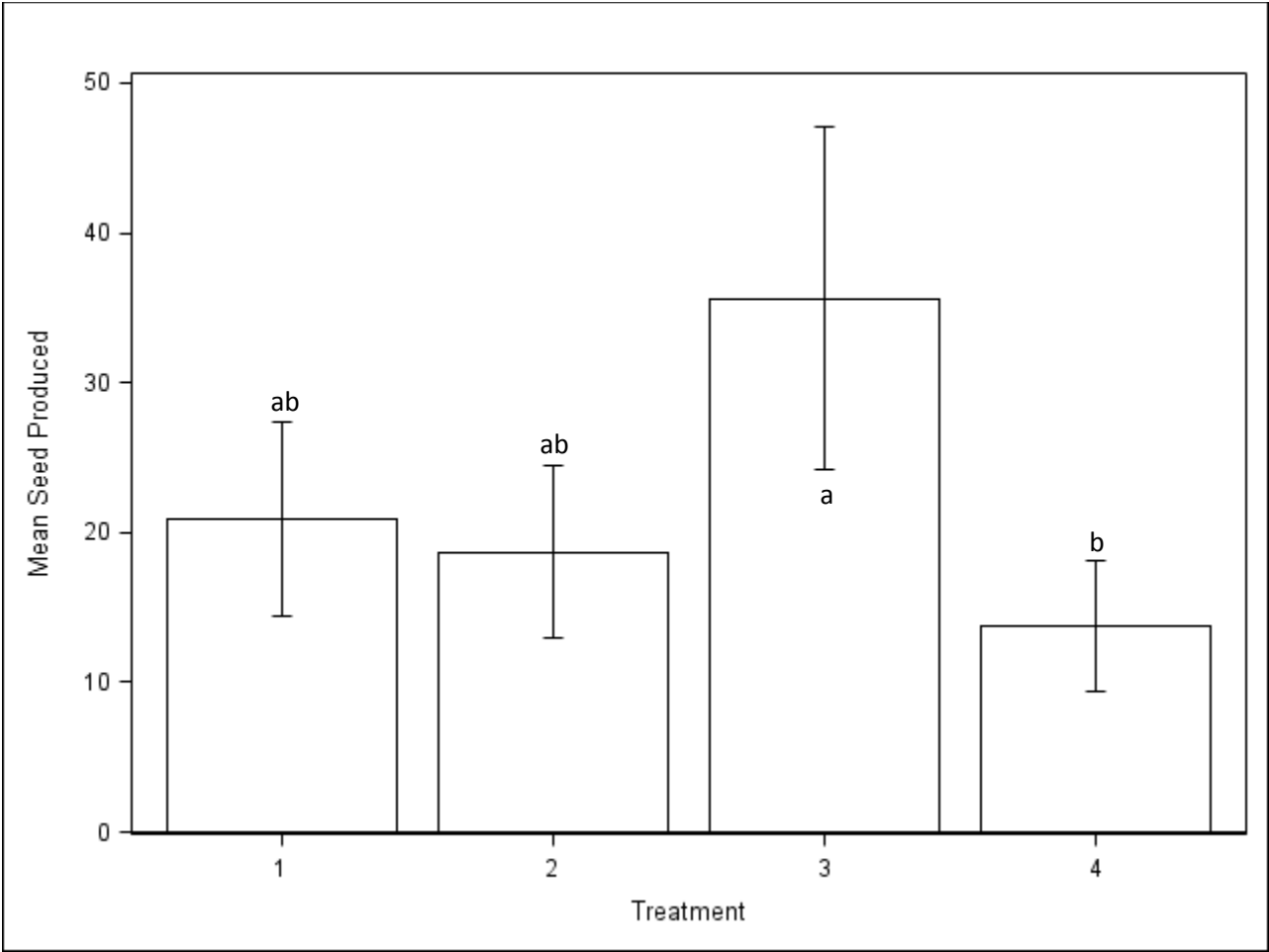


Figure 6.5

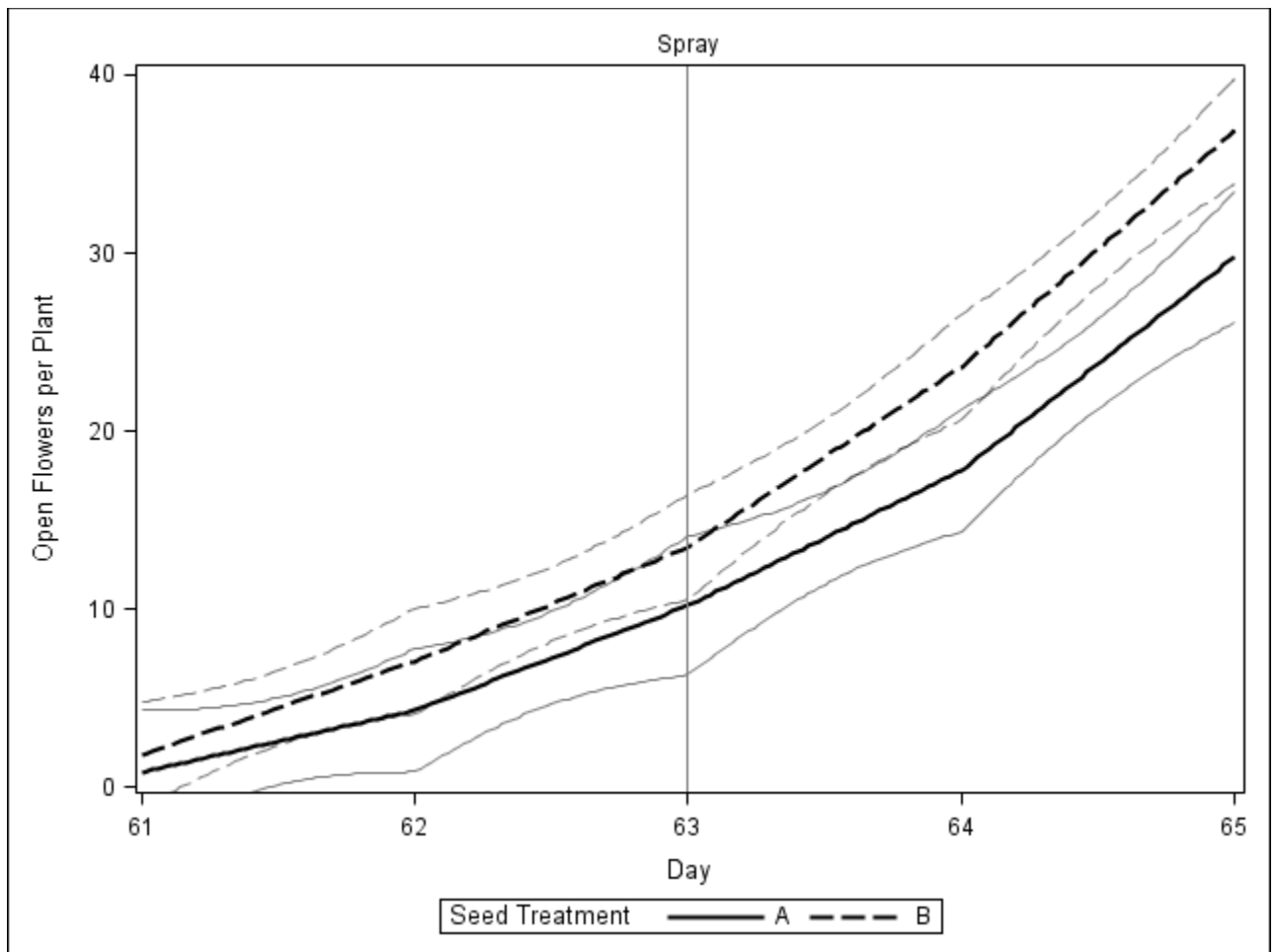
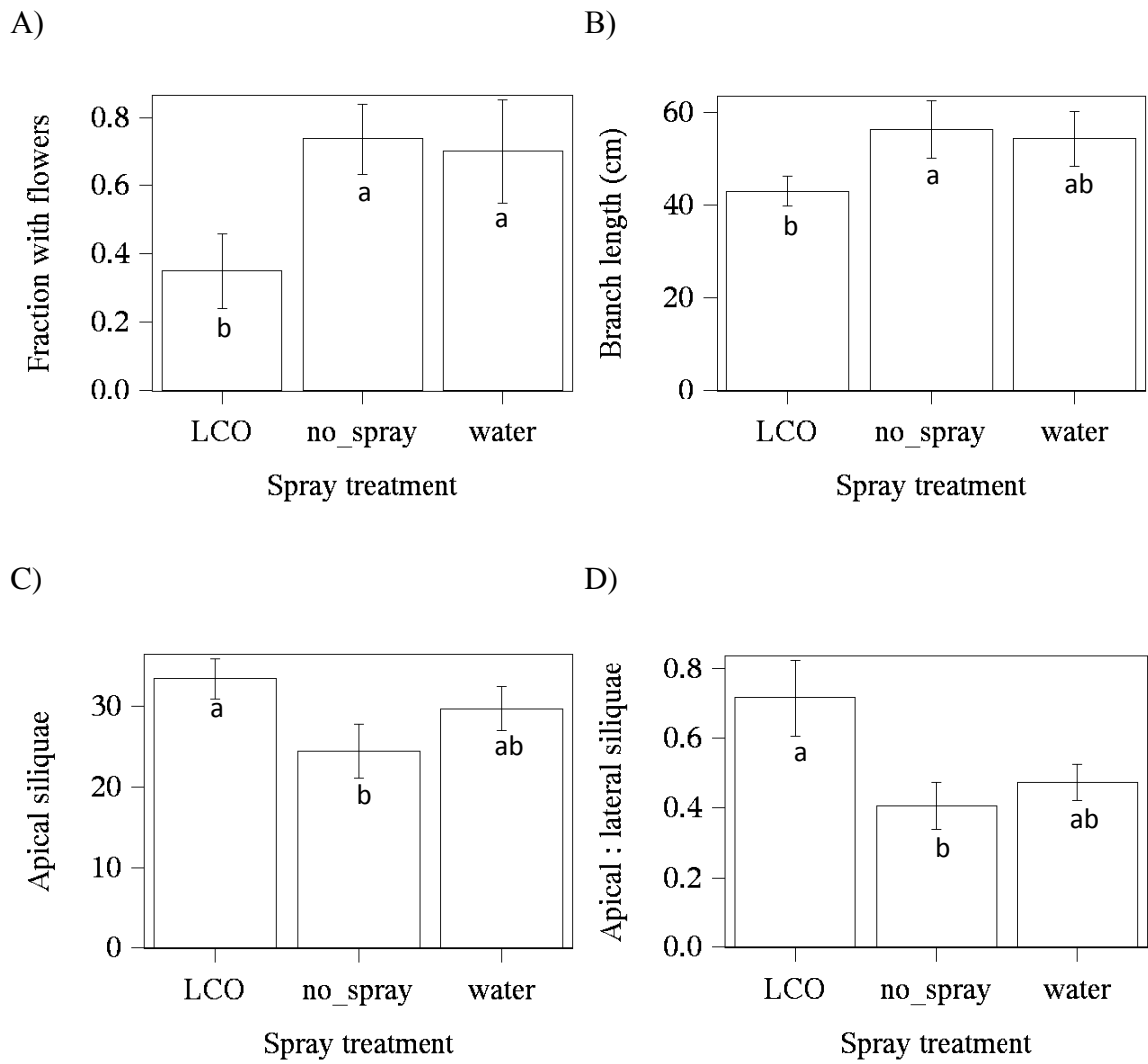


Figure 6.6



## 7 General Discussion

Previous work showed that LCO treatment increased the total SA and ABA contents of *Arabidopsis* rosettes, and treatment with SA alone increased the above ground dry weight of *B. napus* cv. PF plants (Kazemi et al. 2010). Furthermore, abscisic acid is known to have high activity for the induction of oleosin genes (Qi et al. 1998). Lipo chito-oligosaccharide treatment, by increasing total ABA content, may therefore result in the accumulation of oleosins, which can result in relatively small oil bodies, such as are typical of high oil content cultivars (Hu et al. 2009).

Seed weight is one of three direct components of *B. napus* yield on a per plant basis: siliquae per plant, seeds per silique, and seed weight (Diepenbrock 2000; Ali et al. 2003). Plants per unit area could be included as a major factor, when considering yield at the field scale. Some recent work has found seed weight to be negatively and strongly correlated with seed yield potential, *e.g.*, from early-maturing winter rapeseed in South China (Lu et al. 2011; Li et al. 2011). Although, in many studies, all pairwise combinations among the three yield component traits have been found to be negatively correlated, which suggested competition among the sinks for assimilates, seed weight and seed size have been positively correlated with plant productivity and oil and protein content (Shi et al. 2009; Golpavar 2012). Final seed weight is thought to be determined during the last phase in the reproductive cycle, seed filling, by the rate and duration of photoassimilate production, and has been linked to allelic variation in the *B. napus* sucrose transporter gene and its promoter (Barnabás et al. 2008; Li et al. 2011). Although longer siliques produce greater total seed weight than short siliquae, plants with fewer branches and siliquae can distribute more storage substance into each seed with a resulting larger individual seed weight (Norton and Harris 1975).

Escobar et al. (2011) saw significant differences in seed yield, owing to genotype and the interaction of genotype with environment. Seed weight is thought to be more tightly controlled genetically and less influenced by environmental factors than other yield-related traits (Shi et al. 2009). The efforts of breeding have focused generally on improvements of yield rather than tolerance of plants to suboptimal conditions (Barnabás

et al. 2008). Some groups have found that seed weight has high heritability (Ding et al. 2012). From among the genotypes tested by Escobar et al. (2011), the highest seed yield was obtained by hybrid lines. Their open-pollinated cultivar, Goya, yielded seed similarly to the hybrids. Tohidi Moghadam et al. (2011) found high 1000-seed weights were related to the Talaye cultivar. Talaye is known as a high-yield canola cultivar in Iran due to its numerous branches and large seeds. However, Shahin and Valiollah (2009) found that the cultivar factor did not significantly affect the 1000-seed weight of 3 experimental cultivars grown in Iran.

Large seeds normally have better adaptability during germination, and seedlings from large seeds may be superior over ones from small seeds in competitive survival rates (Geritz et al. 1999; Adamski et al. 2009). The advantages of producing large seeds for *B. napus* is that plants grown from large heavy seeds establish better, have higher shoot weights, are more vigorous under stress conditions, and are more tolerant of flea beetle (*Phyllotreta* spp.) damage than seedlings from small light seeds (Bodnaryk and Lamb 1991; Elliot et al. 2008). With each 1.0 g increase in seed weight, improvements in seed yield were greater from years with moist conditions than from a year with dry conditions (Elliot et al. 2008). Plants from small seeds produce the correspondingly lowest seed weight at harvest. Compared with small seeds, large seeds can improve harvest seed weight by 6% (Elliott et al. 2007).

Seed weights have been strongly, positively correlated with the seedling and shoot weights of young Brassica plants (Elliott et al. 2007; Bodnaryk and Lamb 1991); and above ground fresh and dry weights of Topas plants grown in peat pellets at 30 °C were increased for plants treated with  $10^{-9}$  M LCO solutions (Figs. 4.4 and 4.5); and the fresh weights of 04C111 plants that were grown with a chronic supply of  $10^{-11}$  M thuricin, at 10/4 °C, with prohibitively saline conditions ( $200 \times 10^{-3}$  M NaCl), were increased by 58.9 mg, 38 DAP. In light of these facts, further work with these cultivars, under these specific conditions, with these specific treatment concentrations, may yet produce increases to seed yield.

Cultural techniques have been observed to affect seed weight. Leach et al. (1999) observed that very high seeding densities decreased seed dry matter but increased 1000-seed weight for winter cv. Ariana. Sarkar et al. (2007) found the seed produced by *B. napus* under zero-tillage was heavier ha<sup>-1</sup> than under a tillage regime that involved two cross-wise passes with a rotary power tiller. The highest seed yield that they observed was obtained under *O. sativa* straw mulch, as compared to dry water hyacinth (*Eichhornia crassipes* [Kunth.]) mulch and no mulch. However, Sincik et al. (2010) found 1000-seed weight was not affected by either row spacing or seeding rate. There are other abiotic agroecosystem characteristics that affect *B. napus* seed weight. Adequate soil water and well-fertilized soil improve *B. napus* seed and oil yield, in part by increasing seed weight (Tesfamariam 2004; Thomas 2001). Tahir et al. (2007) found that the effect of different irrigation frequency was significant on 1000-seed weight. Higher leaf area index, crop growth rate, and net assimilation rate resulted in more photosynthates, which were stored in the sink/seed, resulting in higher seed weight at higher irrigation frequencies. An increase in seed yield with an increase in the number of irrigations has also been reported by Hati et al. (2001) and Panda et al. (2004). The effect of abiotic fertilization on seed weight has been studied by many groups, for example, Liu et al. (2009) found that the 1000-seed weight was markedly increased by application of molybdenum fertilizer.

Ashraf's and McNeilly's (1990) study indicated that the reason *B. napus* performed better under saline conditions compared to the other brassicas they studied was due, in part, to its relatively high seed weight. Al-Solaimani et al. (2012) found that there was a significant increase in seed weight per plant when plants were irrigated with only mildly saline water, as compared to highly saline water.

High temperatures can disable the ability of *B. napus* to set seed. High temperature conditions can lead to fewer and/or malformed and/or smaller organs in plants in consequence of reduced productivity and impaired development (Barnabás et al. 2008). Morrison (1993) reported *B. napus* cvs. Westar and Delta grown at a daily mean 22 °C in growth cabinets were almost entirely sterile, and abnormal megagametophyte development, owing to high temperature stress, has been observed among *B. napus* plants

at a daily mean 25.5°C (Polowick and Sawhney 1987). Escobar et al. (2011) observed lower seed weights than usual owing to >25 °C temperatures while the *B. napus* plants flowered. Similarly, Istanbuluoglu et al. (2010) observed low 1000-seed weights in 2007 which they attributed to high temperature. Frenck et al. (2011) found elevated temperature reduced seed weight per silique, for the cultivars of *B. napus* they studied: At stressfully high temperatures, biomass accumulation in plants typically declines as a consequence of reduced rates of photosynthesis, reduced light interception due to shortened life cycle caused by faster development, and elevated rates of respiration (Barnabás et al. 2008; Atkin and Tjoelker 2003).

Biotic agroecosystem characteristics also affect *B. napus* seed weight. For example, Ali et al. (2011) found seed weight per silique increased owing to the presence of particular pollinator bees, namely the giant honey bee (*Apis dorsata* [F.]), the red dwarf honey bee (*Apis florea* [F.]), and *Halictus* sp.

Whereas LCO has been observed to increase total plant content of SA and ABA, and ABA has been found to induce *B. napus* oleosin genes, the LCO treatment at an early stage of growth increased seed weight for the rapid cycling plants, but for relatively geriatric Topas plants grown under simulated cool spring conditions, which demanded a longer period of time to reach maturity, the LCO spray at flowering was found to be more effective. These results indicate that timing is important, and it may be relative to the initiation of seed filling. Although a difference in seed weight owing to LCO treatment was not discernible from the experiment in which 04C111 plants were grown under optimal temperature conditions, future experiments should investigate a spray at a later stage of plant development.

## 8 General Conclusions

Lipo-chitooligosaccharide, thuricin 17, and chitopentaose, supplied at the tested concentration levels do not uniformly increase the cumulative germination and growth of *B. napus*. *Brassica napus* cv. Polo seed, treated with a  $10^{-6}$  M concentration of LCO, germinated earlier than the controls, as indicated by the lower slope of its germination curve (Gompertz time constant value,  $b_2$  - Table 3.2). In the 5 to 15 growing degree day period, 75 % more Polo seeds germinated when treated with  $10^{-6}$  M LCO solution than the distilled water controls (Fig 3.1). In the subsequent germination experiments, the experimental *B. napus* genotypes interacted heterogeneously with experimental temperature levels (Tables 3.3 and 3.4). The signal compounds affected *B. napus* germination heterogeneously across the experimental temperature conditions; a  $10^{-6}$  M chitopentaose solution increased the number of seeds that germinated of the experimental *B. napus* cultivars by 17.2 %, under 24 h 30 °C temperature conditions.

In a peat pellet system, at 10/4 °C and 11 DAP, more (+20.8 %) seed that was irrigated with  $10^{-6}$  M LCO solution emerged, as compared to seed irrigated with  $10^{-11}$  M thuricin 17. There were differences due to the experimental treatments in *B.napus* cv. 04C111 leaf numbers and petiole lengths that were visible when comparisons were made across temperature levels (Table 4.1). Regarding plant form, the presence of an adventitious root was significantly and positively correlated to the presence of the LCO treatment (Table 4.2). The evidence provided by the CRCDD experiment lead to the conclusion that irrigating with approximately  $10^{-9}$  M LCO solution provides the greatest effect at 30 °C to produced higher above-ground fresh weights and dry weights of 2-week old Topas plants (Figs. 4.4 and 4.5). However, irrigation with  $10^{-12}$  M LCO solution advanced the Topas plant developmental stage at 17 °C (Fig. 4.6). Evidence provided by growth cabinet experiments, using plant culture vessels, lead to the conclusion that, at 10/4 °C, thuricin 17 treatment increased the tolerance of young 04C111 plants to  $200 \times 10^{-3}$  M NaCl conditions, as indicated by the 58.9 mg elevation of fresh weight, as compared to the controls, 38 DAP. Under 10/4 °C and  $100 \times 10^{-3}$  M NaCl conditions, thuricin 17 treatment produced 04C111 plants with approximately 1 fewer leaves, compared to the controls. The treated plants were therefore relatively oligophyllous. Under 30/30 °C

conditions, the presence of  $10^{-9}$  M thuricin 17 in the agar-based media, in plant culture vessels, eliminated the secondary embryogenesis that otherwise occurred at a rate of 1 extra plant from 10 seeds. Also under 30/30 °C conditions, the effects on plant form due to the experimental concentration of thuricin 17 were discernible at the  $\alpha = 0.05$  level, when leaf number and petiole length were considered across the experimental salinity levels (Fig 4.8 and 4.9).

After one week of growth at 25 °C, which is a relatively high temperature that is prohibitive to seed set, the application of 10 mL  $10^{-6}$  M LCO solution to the seed at planting enhanced rapid cycling *B. napus* emergence by 28.1 %. The seed treatment also increased the proportion of plants that produced seeds by 15.4 % (Fig. 5.1), and increased the yield per plant by 2.009 mg.

Greenhouse experiments showed that LCO-seed-treated 04C111 plants grown to the harvest stage generate approximately 1 more branch, on average, as compared to plants grown from untreated seed. The total branch length of LCO-sprayed 04C111 plants was 16.93 cm longer, and therefore apical dominance was decreased by 17.94 % (Figs. 6.3a and b). The effect of thuricin 17 seed application seems to be relatively disadvantageous: unsprayed 04C111 plants grown from untreated seed in a high temperature greenhouse produced 7 more seeds, on average, than water-sprayed plants grown from seed treated by 10 mL  $10^{-11}$  M thuricin 17 (Fig. 6.4). The application of LCO to Topas seed, which was germinated and grown for 2 months under 10 °C conditions in a greenhouse before step-wise temperature elevation, delayed flowering slightly but significantly from the apex (Figure 6.5). The longest primary branches grown by unsprayed Topas plants were 13.43 cm longer on average than the longest branches produced by LCO-sprayed plants (Fig 6.6b). Topas plants that were sprayed with LCO produced approximately 9 more siliquae, and 76.1 % higher ratios of apical:lateral siliquae, than unsprayed plants (Fig 6.6c and d).

## 9 Summary

To assess the effect of bacteria-to-plant signals as plant growth regulators for new *B. napus* cultivars that were bred for biodiesel production, germination assays were conducted under 10/10, 20/20, 20/30, and 30/30 °C temperature conditions in growth cabinets. Lipo-chitooligosaccharide that had been produced by *Bradyrhizobium japonicum* 532C enhanced the germination of the conventional cultivar Polo, and a  $10^{-6}$  M concentration of the signal stimulated seed germination by 75.0 % in the 5 to 15 GDD period. The experimental 04C111 cultivar germinated successfully and quickly in these assays, under the experimental temperature conditions, as compared to the other cultivars tested. The relatively inhibited germination performance of sister cultivars 02C3 and 02C6 was aggravated by signal treatments. Topas germinated relatively well under 10/10 °C conditions, particularly when treated with LCO. At 30/30 °C, chitopentaose treatment enhanced the germination of all the cultivars, compared to LCO and controls. In peat pellet experiments, irrigation with  $10^{-6}$  M LCO solution stimulated 20.8 % more emergence of 04C111 plants, 11 DAP, compared to thuricin 17-treated seed. Similarly, the application of LCO to fastplant seed stimulated 28.1 % more emergence, compared to water-treated controls, 14 DAP. At 30/30 °C, a  $10^{-6}$  M LCO solution applied to the 04C111 seed at planting produced multifoliate young plants, 11 DAP, as compared to plants grown under lower temperature conditions. However, after two weeks at 17 °C, irrigation with  $10^{-12}$  M LCO solution stimulated Topas plant development. Experiments using Topas indicated that irrigation with near-nanomolar LCO solution would produce the heaviest young plants (Figs. 4.4 and 4.5). Treatment with LCO correlated with adventitious root production by 04C111 plants grown in growth cabinets, but not among the treated plants that were grown in greenhouses. In plant culture vessels, at 10/4 °C and  $100 \times 10^{-3}$  M NaCl, chronic thuricin 17 treatment produced 04C111 plants with 1 less leaf. The reduction of plant surface area by thuricin 17 treatment allowed the plants to tolerate prohibitively saline conditions; at 30/30 °C and  $100 \times 10^{-3}$  M NaCl, chronic treatment with  $10^{-9}$  M thuricin 17 reduced the instances of secondary embryogenesis; and at 30/30 °C, the chronic presence of  $10^{-11}$  M thuricin 17 stunted stem growth as much as  $100 \times 10^{-3}$  M NaCl in the media. In growth cabinet experiments, the  $10^{-6}$  M LCO seed

treatment increased the production of seed by 15.4 % and the final total seed weight per rapid cycling *B. napus* plant by 2.009 mg. The application of thuricin 17 to 04C111 seeds at planting aggravated sterility among plants grown under prohibitively high temperature conditions in a greenhouse experiment (Fig. 6.4). In greenhouse experiments, the LCO seed treatment resulted in *B. napus* plants with ramose forms. A spray of  $10^{-6}$  M LCO solution applied at the flowering stage of plant development stimulated the elongation, by 16.93 cm on average, of the plant total branch length for 04C111, but shortened plant total branch length by 13.43 cm on average for Topas plants, grown under optimal and cool spring temperature conditions, respectively. Treatment with LCO slowed Topas floral initiation slightly but significantly (Fig. 6.5), but the distribution of siliquae was less correlated to plant architecture, and the number of apical siliquae increased by 0.1 per cm of stem, which amounted to approximately 9 more apical siliquae per plant (Fig. 6.6c).

## 10 Suggestions for Future Research

Future work could address the following questions:

1. Irrigation with  $10^{-9}$  M LCO under a controlled 14 h day / 10 h night diurnal cycle at 24 h 30 °C stimulated greater above-ground fresh and dry weight of cv. Topas plants, 14 DAP. Furthermore, irrigation with  $10^{-12}$  M LCO solution advanced Topas plant developmental stage at 17 °C and Topas plants sprayed with  $10^{-6}$  M LCO solution at the flowering stage produced approximately 9 more siliquae, and 76.1 % higher ratios of apical:lateral siliquae. In light of these findings, a greenhouse experiment with controlled environment conditions is proposed to investigate 3 factors: the concentration of LCO, temperature, and the plant developmental stage for application, with levels determined mathematically by the Central Rotatable Composite Design, to confirm the optimal point of interaction that was identified in previous experimentation to increase the number of apical siliquae and oil % d.w for cv. Topas. Stepwise regression of the plant architectural variables could identify the contributing factors to Topas seed quantity and quality under the respective environmental conditions. Separate greenhouses (or translucent containers within a greenhouse) may be used to isolate treated plants, which may have high salicylic acid levels. Isolation of the treatment groups will disallow any stimulation of stress-related pathways by plants responding to the emission of methyl salicylate by their LCO-treated neighbours (Shulaev et al. 1997).
2. Most plant growth regulators currently applied to *B. napus* in agriculture are hormone analogues that can have unintended effects on the health of non-target organisms in the agroecosystem (Wu et al. 2013). Therefore, further experimentation is necessary to determine the effects of LCO on non-target organisms. The effect of LCO application on the growth and competition of a weedy and wild brassicaceous plant such as wild mustard (*Sinapis arvensis* L.), or hoary cress (*Cardaria pubescens* [Meyer] Rollins), or shepherd's-purse (*Capsella bursa-pastoris* [L.] Medic.), or a non-brassicaceous plant such as dandelion

(*Taraxacum officinale* Weber), could be investigated in an experiment similar to the one proposed above.

3. After LCO application to the seed at planting, followed by growth in a greenhouse, 04C111 plants produced approximately 1 more branch, as compared to plants grown from untreated seed. The resultant sum length of branches produced by LCO seed-treated plants was 117 % longer on average. Therefore, a greenhouse experiment is proposed, with a design as described in Section 6.3, to investigate the characteristics of LCO-stimulated 04C111 branch growth. To look at the effect of these agents on abscisic acid and indole-3-acetic acid, enzyme-linked immunosorbent assay test kits (Phytodetek, Agdia Inc., Elkhart, Indiana, USA) could be used (*e.g.*, Pallai et al. 2012). This experiment would determine whether internode elongation or the production of new nodes is the cause of branch lengthening, and whether or not LCO seed-treatment results in an expansion of 04C111's leaf area, which could be determined nondestructively using a LI-3000C Portable Area Meter (LI-COR, Lincoln, Nebraska, USA). Internode elongation, the number of nodes, and leaf area may be factors that contribute to yield under particular agricultural conditions. Alternatively, they may be factors that contribute to lodging, which would be detrimental to yield, *e.g.*, in Western Canadian agriculture. Internodes and nodes of plants would be counted at regular intervals during the time of growth. Poisson distribution would be specified for count data using PROC GLIMMIX in SAS, and the Type III Test of Fixed Effects will establish whether or not the treatment has an effect on counts. A variety of curve functions could fit the count and area data using PROC NLIN in SAS, and the most appropriate functions would be selected based on low mean square error statistics.
4. Jung et al (2011) found that thuricin 17 induced plant defense-related enzymes such as ascorbate peroxidase in soybean (*Glycine max* [L.] Merr. cv. OAC Bayfield), and Suzuki et al. (2013) found that Arabidopsis plants lacking ascorbate peroxidase 2 produced more seeds under prolonged heat stress. The results reported above indicated that unsprayed *B. napus* plants grown from untreated

seed in a high temperature greenhouse produced ~7 more seeds, which was a 51.44 % increase, compared to water-sprayed plants grown from seed to which 10 mL  $10^{-11}$  M thuricin 17 was applied at planting (Fig. 6.4). The question remains, does thuricin 17 induce ascorbate peroxidase in *B. napus*, thereby inhibiting hydrogen peroxide signaling that would otherwise induce the protection of reproductive tissue under heat stress conditions? Plants would be grown according to the method of the original experiment (Section 6.4.2), reproductive material would be harvested at repeated time points, and measurements of enzymatic reactions of ascorbate peroxidase could be done using a method similar to that of Stasolla and Yeung (2001).

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