J Plant Growth Regul (2015) 34:183–195
DOI 10.1007/s00344-014-9456-7

The Plant Growth Regulator Lipo-chitooligosaccharide (LCO) Enhances the Germination of Canola (*Brassica napus* [L.])

Timothy Schwinghamer · Alfred Souleimanov · Pierre Dutilleul · Donald Smith

Received: 14 June 2014 / Accepted: 21 August 2014 / Published online: 16 October 2014

Abstract In agricultural environments where canola (*Brassica napus*) is grown, slow germination can increase the susceptibility of seedlings to pathogens, delay maturity, and decrease yield. Bacterial products that enhance germination have been 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 the undecorated chitin backbone of LCO. Gompertz functions were estimated and used for inferences regarding the signal, cultivar-by-temperature, and signal-by-temperature effects on 6 cultivars (02C3, 02C6, 04C111, 04C204, Polo, and Topas). LCO (10^{-6} M) was found to increase Polo germination by 75.0 %, during the 5–15 growing degree day period. Such early *B. napus* germination can, under field conditions, increase canopy coverage and yield. Further experimentation with the other experimental cultivars discerned an improvement in the germination of Topas, following treatment with LCO, under ideal (24 h 25 °C) and abiotic stress (24 h 10 °C) growing conditions, as compared to Polo and 04C204. The response to LCO was discernable for Polo under AOSA (J Seed Technol 16:112, 1993) standard temperature conditions and for Topas when considered across temperature conditions in comparison to Polo and 04C204.

T. Schwinghamer · A. Souleimanov · P. Dutilleul · D. Smith (✉)
Department of Plant Science, McGill University, Macdonald Campus, 21111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec H9X 3V9, Canada
e-mail: donald.smith@mcgill.ca

Keywords *Brassica napus* · Canola · Chitopentaose · Lipo-chitooligosaccharide · Thuricin 17 · Germination · Abiotic stress

Introduction

Rhizobia-legume and rhizobia-*Parasponia* symbiotic interactions are mediated by lipo-chitooligosaccharide signal molecules (reviewed by Oldroyd and others 2010; Op den Camp and others 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 and others 2010). The Nod factor we applied was Nod Bj V (C18:1, MeFuc) (MW 1,415 Da). It is a fatty acylated chitin pentasaccharide, that is, five *N*-acetylglucosamine (GlcNAc) residues with a 2-*O*-methyl-L-fucosyl residue attached to the 6-position of the reducing-end GlcNAc (Carlson and others 1993). The relative orientation of the oligosaccharide and acyl moieties can range from being extended to essentially perpendicular to being quasi-parallel (Groves and others 2005; Morando and others 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 and others 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 and others 2000).

Brassica napus has neither symbiotic rhizobial nor mycorrhizal associations, but plants that do not host rhizobia may nevertheless perceive LCO (Liang and others 2013). 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 and others 2010). (Ohnuma and others 2008) showed that the binding affinities of the LysM domains increase as the number of GlcNAc oligomer repeats increases. However, Liang and others (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 2014). These levels of salicylic acid (SA) accumulation are somewhat less than the 100–200 % increase over background levels that would be expected to be induced at the primary leaf due to second stage systemic acquired resistance (SAR) (Cameron and others 1999), and although 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 2014). Previous work has shown that Nod Bj V(C18:1, MeFuc) from *Bradyrhizobium 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 and others 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 and others 2003). Lipo-chitooligosaccharides from different rhizobia added to tomato (*Lycopersicon esculentum* [Mill.]) cell culture suspensions at 10^{-8} M stimulated a rapid transient alkalinization of the medium (Staehelin and others 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 and others 1993).

Chitopentaose is a linear polysaccharide composed of (1 → 4)-linked GlcNAc residues derived from chitin. Chitin is present in the exoskeleton of arthropods, and fungal cell walls (Rinaudo 2006). Chito-oligosaccharides are MAMPs that may be perceived by plants. 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 and others 2008). For example, 10^{-8} M chitopentaose, produced by glomeromycete *Rhizophagus irregularis*, triggered maximal Ca^{2+} spiking in barrel medic (*Medicago truncatula* [Gaertn.]) root organ culture atrichoblast cells (Genre and others 2013). Wang and others (2010) produced chitopentaose, among other things, after 5 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 and others (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, chitopentaose has been shown to promote growth of radish (*Raphanus sativus* [L.]) (Tsugita and others 1993 cited in Ohta and others 2000 [in Japanese]) and enhance the germination of wheat (*Triticum aestivum* [L.]) and maize (*Zea mays* [L.]) (Guan and others 2009).

Bacillus thuringiensis non-*Bradyrhizobium* endophytic bacterium 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 and others 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 and others 2011). Sequences for the peptide were published by Lee and others (2009). Peptides such as thuricin 17 could generate or amplify messages, but can act as signals themselves within the phloem (Giavalisco and others 2006). Previous work has shown that thuricin 17 can enhance soybean (*Glycine max* [L.] Merr.) and corn (*Z. mays* L.) growth (Lee and others 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 and generalized logistic functions for the cultivars of interest, which were supplied with LCO, thuricin 17, and chitopentaose in solution. The second objective was to

Table 1 The seed oil content of the experimental cultivars (±2 % d.w.)

Brassica napus cultivar	Seed oil content (% d.w.)[a]
02C3	50.6
02C6	51.2
04C111	53.8
04C204	51.0
Polo	47.2
Topas	42.2

[a] University of Manitoba agronomic data

determine whether or not the interaction effects of cultivar-by-temperature and signal-by-temperature had effects on the function parameters, and indices derived from them. The potential plant growth regulators evaluated here have been shown to affect seed germination, but there was until now little data regarding canola and no information regarding potential effects on new, high oil content cultivars of *B. napus*; in light of Linder's (1998) report, that the modification of *B. napus* seed oil can induce dormancy and inhibit germination, this requires investigation.

Materials and Methods

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 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 indicate previous crosses, here: HiQ////Apollo///86LL141//Tatyoon/R83-14. The crosses that produced sister cvs. 02C3 and 02C6 are also presented in standard breeder's notation, here: Polo/// 86LL141//Tatyoon/R83-14. *Brassica napus* cv. Polo is a conventional, double low, that is, 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 because of its relatively low seed oil content.

Production of Lipo-chitooligosaccharide

Bradyrhizobium japonicum strain 532C was cultured in the Yeast Extract Mannitol (YEM) medium (Mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4$ 7·H_2O 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., Mariolta, OH, USA) at 150 rpm and 27 ± 2 °C. When the OD_{620} reached 0.4–0.5 (4–5 days), genistein (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was added to a final concentration of 5×10^{-6} M and the culture was incubated for an additional 48 h. The 2 L culture was extracted with 0.4 volume of HPLC grade 1-butanol by shaking vigorously for 10–15 min and then allowing the material to stand overnight. The organic fraction was then separated and completely dried under vacuum, in a rotary evaporator (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_{18} reverse-phase column (Vydac, Hesperia, CA, USA; catalog # 218TP54) with a flow rate of 1.0 mL min^{-1} and a Vydac guard column at wavelength 214 nm. As a baseline, 18 % ACN (ACN/H_2O; 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 18–82 % ACN. The LCO eluted at 84–86 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 (Novalyphe Freeze Drying system, model SNL216 V, Savant Inc., Holbrook, USA) and stored at −20 °C until further use (Souleimanov and others 2002a).

Production of Thuricin 17

Bacillus thuringiensis NEB17 was cultured in King's medium B (Proteose peptone no. 3 20 g, K_2HPO_4 0.66 g, $MgSO_4$ 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 and others 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^{-1}) (catalog 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 and others 2002). Preparative HPLC samples were separated into 120-min fractions and were analyzed for peaks with retention times between 80 and 82 min as this is when the peptide elutes. The peptide elutes in approximately 60 % ACN. This material is denoted purified bacterial peptide and samples were lyophilized and stored at −20 °C.

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 rinsed through agitation in distilled water. We conducted our research 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 cabinet conditions were 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). For Phase 2, growth cabinets were also set to the ideal germination temperature (25 °C) (Kucera and others 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 (Omidi and others 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 and others 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 and others 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 level was assessed every 6 h.

Table 2 Bayesian information criteria (BICs) for selected covariance structures

TYPE option	Phase 1	Phase 2	Phase 3
Sp(exp)(GDD)[a]	5,368.40	6,550.48	19,774.88
Sp(powa)(GDD)	5,368.40	6,550.48	19,774.89
Ar(1)	5,351.20	6,550.48	19,774.80
Arh(1)	5,351.20	6,403.29	19,778.34
Un	5,368.39	6,410.42	19,776.00
Cs	5,351.20	6,550.48	19,774.89
Csh	5,351.20	6,403.29	19,778.34
Toep	5,368.40	[b]	19,774.80
Toeph	5,351.20	6,403.29	19,778.34
Ante(1)	5,368.40	6,410.42	19,778.34

[a] GDD indicates growing degree days

[b] QUANEW Optimization could not be completed. This indicated that a simpler model would be more appropriate

Statistical Analysis

Germination data were analyzed with generalized linear mixed models, using SAS PROC GLIMMIX (SAS 9.3,

Table 3 Mean square errors for the comparison of nonlinear regression functions fitting cumulative germination counts for the three phases of the experiment

Model function	Equation	Mean square error		
		Phase 1	Phase 2	Phase 3
Exponential	AB^X	55.217	57.85	26.569
Generalized logistic	$\frac{A}{(D+e^{(B-CX)})}$	5.2199	30.414	18.263
Logisitic	$\frac{A}{(1+e^{(B-CX)})}$	5.2199	30.414	18.263
Gompertz	$b_0 \times e^{[-e^{[-b_1-b_2\times GDD]}]}$	5.2091	30.987	18.14

SAS Institute Inc., Cary, NC, USA). Binomial distribution and the logit link function were specified. GDD was nested within the experimental repetition. Growing degree days were calculated based on this equation:

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

A variety of covariance structures were assessed based on the Bayesian information criteria (BICs) (Table 2). The best covariance structures for the respective experimental phases were selected. For Phase 1, autoregressive, AR(1), and compound symmetric, CS, were equally suitable, based on the BICs. The CS structure, which has constant variance and constant covariance, arises naturally with nested random effects. Therefore, the CS option was selected. The compound symmetry structure with heterogeneous variances, CSH, was selected for Phase 2. A low BIC indicated that the AR(1) structure was the most appropriate for the Phase 3 data.

A series of functions for nonlinear regression were fitted to the data using SAS PROC NLIN code by Sit and Poulin-Costello (1994) (Table 3). Based on the low mean square errors (Table 3), Phase 1 and Phase 3 data were described well by the Gompertz equation:

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

where *GDD* is growing degree days; *E* is cumulative germination; b_0 is asymptotic maximum of *E* taken as the maximum cumulative germination; b_1 is empirical Gompertz shape parameter; and b_2 is the slope of the curve, the Gompertz time constant.

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

is the germination accumulated by thermal time GDD_i, at the Gompertz inflection point (Piegorsch and Bailer 2005). The differences between Gompertz parameters were assessed based on 95 % confidence intervals computed by SAS PROC NLIN. Phase 2 data were assessed using generalized logistic functions. 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's adjustment for multiple comparisons. Where the number of levels was above 6, Scheffé's single-step adjustment was used.

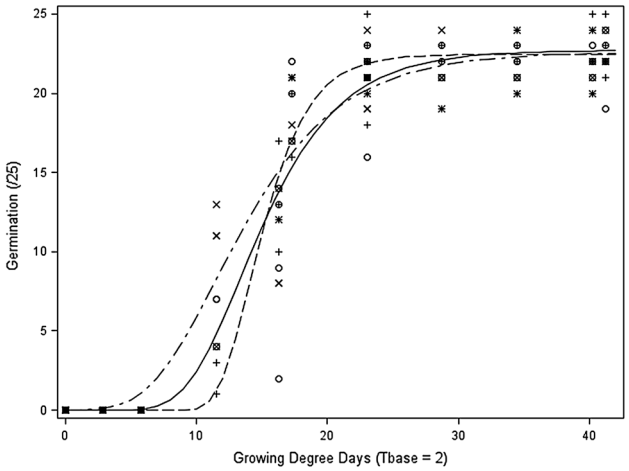

Fig. 1 Gompertz curves $\left(E = b_0 \times e^{\left[-e^{[-b_1-b_2\times GDD]}\right]}\right)$ overlaying a scatterplot showing the numbers of *B. napus* cv. Polo seeds germinated. The 95 % Confidence Limits for Gompertz parameters, calculated for seeds treated with a 10^{-6} M concentration of LCO (*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* ($R^2 = 0.94$)

SAS PROC UNIVARIATE was used to assess the distribution of the parameters and derived indices. We used SAS PROC MIXED to make comparisons when the normality condition was satisfied. When the parameters or derived indices did not conform to the conventional statistical assumptions, nonparametric Kruskal–Wallis tests, with adjustments for multiple comparisons, were used, that is, Dunn's tests (Elliot and Hynan 2010).

Only differences at the $p \leq 0.05$ level were considered significant. For the early germination data from Phase 1, SAS PROC UNIVARIATE was used to generate *n* values, means (μ), standard errors (se), but in other cases, back-transformed, inverse-linked means were produced using the ILINK option. 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.

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. 1).

Table 4 The Gompertz function parameters estimated from Phase 1 data using SAS PROC NLIN that describe the germination of *Brassica napus* cv. Polo at AOSA (1993) standard conditions

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

Considering the nonlinear regression on Polo germination in Phase 1, the Gompertz parameters for seed treated with LCO were different from the control ($p < 0.05$) (Table 4). 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) ($p = 0.0177$). However, considering the experimental factors in the generalized linear mixed model, only the GDD had an effect ($p < 0.0001$).

Polo seed treated with 10^{-6} M LCO germinated earlier than the controls, as indicated by the lower value of the slope of its germination curve (b_2) (Table 3). Lipo-chitooligosaccharide concentration 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 LCO treatments caused a later initiation of germination, however, the seeds germinated more uniformly and to a final level closer to 100 %, 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 5–15 GDD early germination period, 75 % more LCO-treated Polo seed germinated, of 25 seeds per Petri plate, compared to the water-treated controls ($n_{obs} = 64$; $\mu_{\mu M\ LCO} = 4.9$ seeds, se = 2.1; $\mu_{\mu M\ chito} = 2.8$ seeds, se = 1.1; $\mu_{H_2O} = 0.8$ seeds, se = 0.4; unadjusted $p = 0.0007$).

Based on the inverse-linked means for the Phase 2 data, Topas seeds treated with LCO exhibited 167 % more germination than water-treated Polo ($p < 0.0001$), and 242 % more germination than water-treated 04C204 ($p < 0.0001$). The back-transformed least squares means for the numbers of germinated seeds are shown in Table 5 and Fig. 2. The estimates for the parameters of the generalized logistic functions for Phase 2 data, and the subsequently calculated horizontal asymptotes and inflection points for the curves, are shown in Table 5.

The comparison of cultivars at the $\alpha = 0.05$ level provided evidence of location differences for the function parameters *A*, *B*, and *C*, and for the derived horizontal asymptote and inflection point. With respect to the values of *A*, which correspond to the heights of the generalized logistic curves: Polo > Topas > 04C204; and LCO > distilled water (Table 5). With respect to the values of *B*, for which higher values indicate a deeper concavity for the curve: 04C204 > Topas > Polo; and LCO > chitopentaose > distilled water. With respect to the values of *C*, which corresponds to the steepness of the curve: 04C204 > Topas > Polo; and LCO = chitopentaose > distilled water. With respect to the horizontal asymptote as $X \rightarrow \infty$: Polo > Topas > 04C204; and LCO > distilled water. The generalized logistic function is symmetric about the inflection point, where 04C204 > Polo > Topas; and LCO = chitopentaose > distilled water. The comparison between the 2 temperature levels at the $\alpha = 0.05$ level provided evidence of significant location differences, for the function parameters and indices derived from them.

For Phase 3, the single step Scheffé adjustment for multiple comparisons does not require the Type 3 Test of Fixed Effects to be assessed before referring to the *t* test, but the contribution of the experimental factors and their interactions may be assessed (Table 6). The inverse-linked least squares means are shown in Figs. 4 and 5 and Tables 7 and 9.

T-tests with Scheffé's adjustment indicated that, at continuous (for 24 h) 10 °C temperature conditions, 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 ($p < 0.05$) (Fig. 3; Table 8). At continuous 10 °C, 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. 4; Table 8).

Consistently, at 10 °C, 02C3 had fewer seeds germinated than 04C111, 04C204, and Polo ($p < 0.0001$) and in comparison to Topas ($p = 0.0001$). Similarly, 02C6 counts were lower than 04C111, 04C204, and Polo ($p < 0.0001$). At 20 °C, 04C111 had more seeds germinated than 02C3, 02C6, 04C204, and Topas ($p < 0.001$). 02C3 had fewer seeds germinated than Polo ($p < 0.0001$); and 02C6 had

Table 5 Cultivar-by-treatment least squares means for germinated seeds, counted from 25 seeds per plate, and approximate 95 % confidence limits for the parameters[a] of generalized logistic functions estimated by SAS PROC NLIN

Cultivar	Signal	Mean ± SE	Approximate 95 % Confidence Limits						Horizontal asymptote at $X \to \infty$	Inflection point ($s = B/C$)
			A		*B*		*C*			
			LL	UL	LL	UL	LL	UL		
04C204	Distilled water	0.2667 ± 0.02428^{Cb}	33.3744	37.0814	4.6593	6.9479	0.2293	0.3715	17.6139	19.3173
	LCO	0.2744 ± 0.02495^{C}	31.0230	34.4866	5.3436	8.5598	0.2598	0.4515	16.3774	19.5478
	Chitopentaose	0.2704 ± 0.02478^{C}	32.4537	36.7239	5.2695	9.1104	0.2460	0.4689	17.2944	20.1139
Polo	Distilled water	0.3867 ± 0.02958^{BC}	41.3345	49.9914	2.9361	4.4804	0.1369	0.2499	22.8315	19.1714
	LCO	0.3827 ± 0.03012^{BC}	41.1940	49.9322	3.0639	4.7802	0.1402	0.2615	22.7816	19.5257
	Chitopentaose	0.4185 ± 0.03099^{BC}	41.3142	50.0446	3.0012	4.6963	0.1425	0.2662	22.8397	18.8348
Topas	Distilled water	0.5037 ± 0.03166^{AB}	40.2983	48.5206	2.9260	4.7732	0.1667	0.3215	22.2047	15.7715
	LCO	0.6462 ± 0.03185^{A}	42.3173	50.1126	3.1809	5.1313	0.1869	0.3479	23.1075	15.5435
	Chitopentaose	0.5299 ± 0.03178^{AB}	41.4639	48.8484	3.1919	5.0069	0.1829	0.3297	22.5781	15.9940

[a] The *D* parameter for the generalized logistic function was uniformly estimated to be 2.0

[b] Superscripts indicate difference at the $p < 0.005$ level

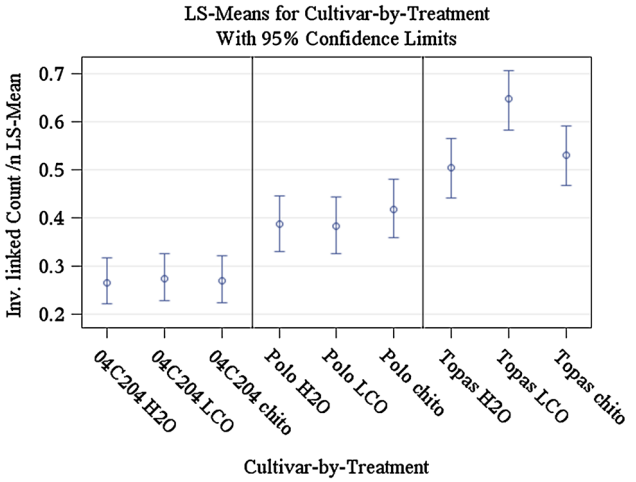

Fig. 2 Inverse-linked least square means of germination counts, out of 25 seeds per plate, for levels of the cultivar-by-treatment interaction from Phase 2. The levels of treatment are indicated by "H_2O"—water; "LCO"—10^{-6} M lipo-chitooligosaccharide; "chito"—10^{-6} M chitopentaose

Table 6 Type 3 tests of fixed effects for phase 3

Effect	Num DF	F Value	Pr > F
cv	5	47.96	<.0001
trt	3	1.64	0.1782
cv*trt	15	0.54	0.9207
dayT	2	624.89	<.0001
dayT*cv	10	11.68	<.0001
dayT*trt	6	0.74	0.6191
dayT*cv*trt	30	0.69	0.8943
GDD	1	10,809.1	<.0001
GDD*cv	5	78.18	<.0001
GDD*trt	3	0.99	0.3962
GDD*cv*trt	15	0.94	0.5159
GDD*dayT	2	678.09	<.0001
GDD*dayT*cv	10	3.22	0.0004
GDD*dayT*trt	6	1.58	0.1493
GDD*dayT*cv*trt	30	0.79	0.7783

cv Cultivar, *trt* treatment, *dayT* daytime temperature, *GDD* growing degree day

fewer seeds germinated than Polo ($p = 0.0013$). At 30 °C, 02C3 had fewer seeds germinated than 04C111, 04C204, and Polo ($p < 0.0001$), and in comparison to Topas ($p = 0.0015$). Again similarly, at 30 °C, 02C6 counts were lower than 04C111 ($p = 0.0087$), 04C204 ($p = 0.0030$), Polo ($p = 0.0046$).

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 at continuous 20 °C, and Polo also had a higher E_i than 02C3 at continuous 20 °C (Dunn's test, $\alpha = 0.05$) (Fig. 3; Table 8). Higher E_i values for 04C111 and Polo at continuous 20 °C indicated abundant radicle emergence and survival for these cultivars (Fig. 3; Table 8).

T-tests with Scheffé's adjustment indicated that, at continuous 30 °C, 02C3 had a lower E_i than 04C111, 04C 204, Polo, and Topas (Table 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; Table 8).

Table 7 Differences between least squares means for germinated seeds counted out of 25 seeds per plate, for the levels of the cultivar × temperature interaction

Cultivar	Day temperature (°C)	Mean ± SE
04C111	20	0.5729 ± 0.02227^{Aa}
Polo	20	0.4453 ± 0.02192^{BA}
Polo	10	0.3475 ± 0.02027^{BC}
04C204	10	0.3465 ± 0.02004^{BC}
04C111	10	0.3368 ± 0.02051^{BC}
Topas	20	0.3071 ± 0.01903^{BC}
04C204	20	0.2701 ± 0.0177^{DC}
02C6	20	0.265 ± 0.01751^{DC}
Topas	10	0.2474 ± 0.01691^{DCE}
04C204	30	0.2207 ± 0.0162^{DCE}
Polo	30	0.2184 ± 0.0162^{DCE}
02C3	20	0.2171 ± 0.01548^{DCE}
04C111	30	0.2164 ± 0.01667^{DCE}
Topas	30	0.1575 ± 0.01311^{DFE}
02C6	10	0.1434 ± 0.01153^{FE}
02C3	10	0.117 ± 0.00994^{GF}
02C6	30	0.1068 ± 0.009982^{GF}
02C3	30	0.06734 ± 0.007182^{G}

[a] Scheffé Grouping for day temperature × cultivar Least Squares Means ($\alpha = 0.05$). LS-means with the same letter are not significantly different

With respect to the cultivar-by-treatment interaction, the highest germination mean (~10.0 seeds germinated) was observed for 04C111 treated with thuricin 17 and the lowest (~2.8 seeds germinated) was observed for 02C3 treated with LCO. The complete lists for the inverse-linked least squares means for the levels of the cultivar-by-treatment interaction are shown in Table 9 and Fig. 5.

Discussion

Under typical agricultural field conditions, early germination is an important factor that affects the yield of annual varieties of canola (*B. napus* [L.]) in the northern temperate areas such as western Canada. Owing to a very short growing season, particularly in the prairie regions, *B. 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 1–2 weeks. Furthermore, *B. napus* must be planted relatively deeply to establish successfully under dry soil surface conditions (Harker and others 2012). Owing to these and other factors, only 50 % of planted *B. napus* seeds typically emerge under agricultural conditions (Harker and others 2003). Reduced germination rates can increase the susceptibility of seedlings to soil borne pathogens, for example, 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 and others 2011).

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, for example, 25 °C, for rapid germination (Kucera and others 2005). Previous work has indicated that cool (≤10 °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 and others 2005; Achard and others 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 and others 2005; Doherty and others 2009). *BN115*, a gene related to an Abscisic 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 and others 2001; Chen and others 2011; Zhuang and others 2011).

The challenges to *B. napus* germination are discussed below; they include light, lack of moisture, salinity, seed characteristics, soil compaction, and temperature conditions (Shaykewich 1973; Farhoudi and others 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. 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 1998). The negative effect of seed oil modification reported by Linder (1998) may be pertain to 04C204's poor germination performance under continuous 25 °C conditions, and this is discussed below. Although Timiriaziff (cited by Coffman 1923) reported the germination of brassicaceous seed in a block of ice, the optimum temperatures for *B.*

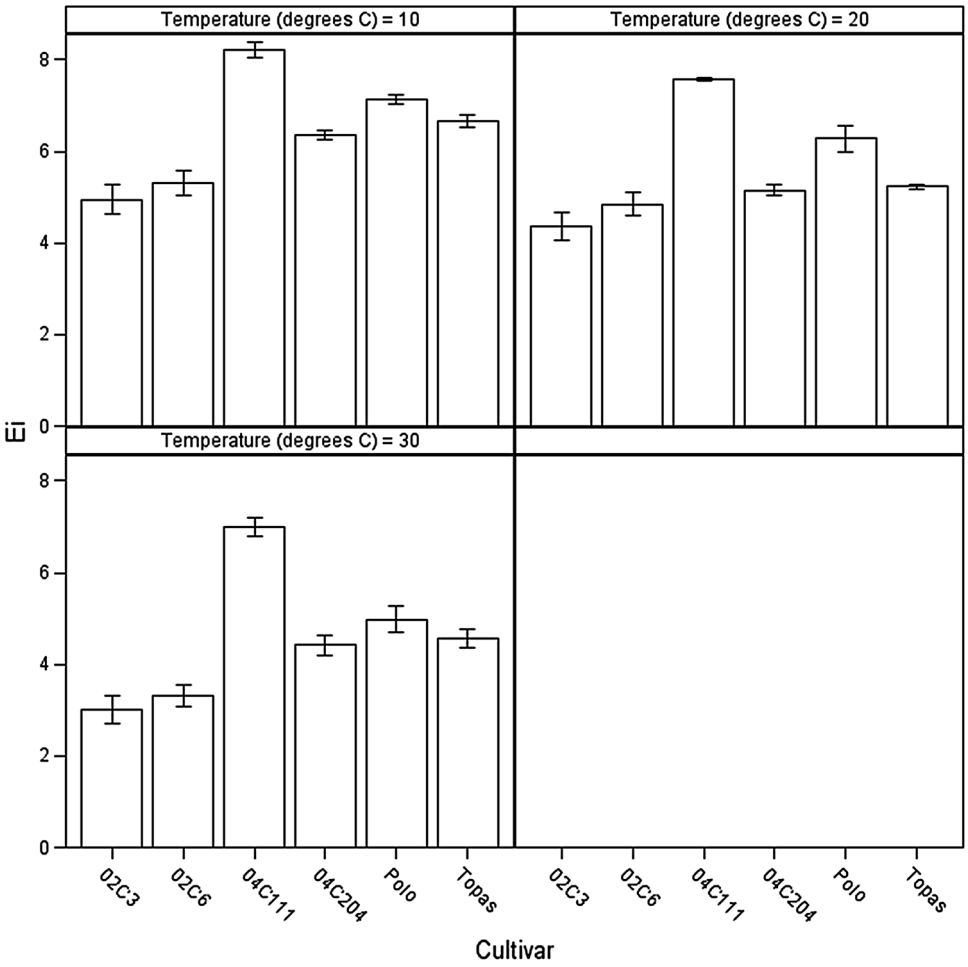

Fig. 3 Accumulated germination at Gompertz function inflection points (E_i). *Bars* indicate standard errors

Table 8 The mean germination accumulated near the midpoint of germination ($E_i \pm$ standard error) for the experimental cultivars, under controlled temperature conditions

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

Superscripts indicate least significant difference ($p < 0.05$) (Saxton 1998)

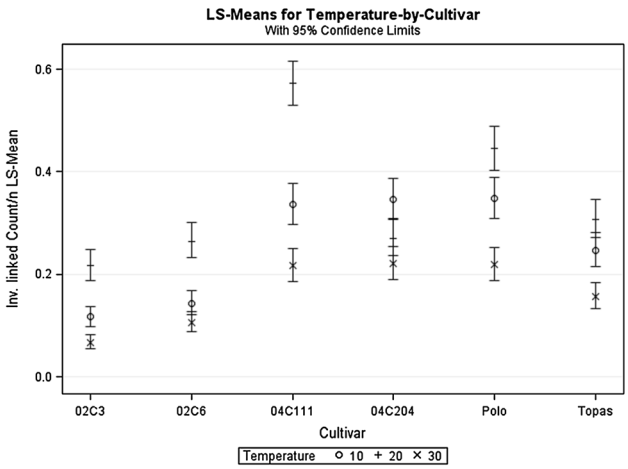

Fig. 4 Inverse-linked least squares means for germination counts, out of 25 seeds per plate, for levels of the temperature-by-cultivar interaction from Phase 3. The levels of cultivar are placed on the horizontal axis

napus are in the range of 10–30 °C. Low temperature conditions slow the rate of imbibition, damage *B. napus* embryos by chilling injury, and prevent germination, which lead to lower frequencies of radicle emergence and even to death (Omidi and others 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

Table 9 Inverse-linked least squares means for germinated seeds counted, out of 25 seeds per plate, for levels of the cultivar × treatment interaction

Cultivar	Treatment	Mean ± SE
02C3	H_2O	0.1229 ± 0.01258^{GFa}
02C3	LCO	$0.1123 \pm 0.01176^{DEFG}$
02C3	Thuricin 17	0.1175 ± 0.01222^{FG}
02C3	Chitopentaose	0.1347 ± 0.01329^{EFG}
02C6	H_2O	$0.1581 \pm 0.01481^{CDEFG}$
02C6	LCO	$0.1466 \pm 0.01412^{DEFG}$
02C6	Thuricin 17	$0.158 \pm 0.01495^{CDEFG}$
02C6	Chitopentaose	$0.1872 \pm 0.01649^{BCDEFG}$
04C111	H_2O	0.312 ± 0.02348^{ABC}
04C111	LCO	0.3531 ± 0.02478^{AB}
04C111	Thuricin 17	0.3997 ± 0.02568^{A}
04C111	Chitopentaose	0.3955 ± 0.02583^{A}
04C204	H_2O	$0.2815 \pm 0.02117^{ABCD}$
04C204	LCO	$0.2806 \pm 0.02126^{ABCDE}$
04C204	Thuricin 17	$0.2518 \pm 0.01983^{ABCDEF}$
04C204	Chitopentaose	$0.2919 \pm 0.02158^{ABCD}$
Polo	H_2O	0.3536 ± 0.02403^{AB}
Polo	LCO	0.3032 ± 0.02224^{ABC}
Polo	Thuricin 17	0.3226 ± 0.02305^{AB}
Polo	Chitopentaose	0.3418 ± 0.02356^{AB}
Topas	H_2O	$0.216 \pm 0.01828^{ABCDEFG}$
Topas	LCO	$0.2242 \pm 0.01882^{ABCDEFG}$
Topas	Thuricin 17	$0.2474 \pm 0.01991^{ABCDEF}$
Topas	Chitopentaose	$0.2386 \pm 0.01943^{ABCDEFG}$

Scheffé grouping for cultivar × treatment least squares means ($\alpha = 0.05$). LS-means with the same letter are not significantly different

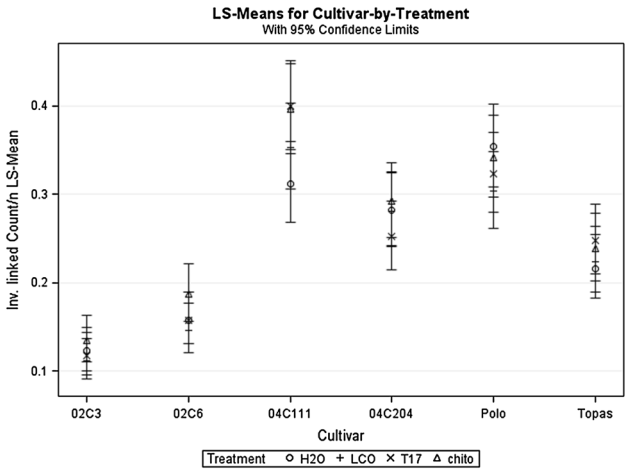

Fig. 5 Inverse-linked least squares means for germination counts, out of 25 seeds per plate, for levels of the cultivar-by-treatment interaction from Phase 3. The levels of treatment are indicated by "H_2O"—water; "LCO"—10^{-6} M lipo-chitooligosaccharide; "chito"—10^{-6} M chitopentaose

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 and others 1983; Brown and Morra 1996).

These results agree with previous work that found that LCO can play a significant role in seed germination (Souleimanov and others 2002b). Work done by this group has shown that LCO enhances the germination of *Arabidopsis*, common bean (*Phaseolus vulgaris* [L.]), beet (*Beta vulgaris* [L.]), cotton (*Gossypium hirsutum* [L.]), soybean (*G. max*), rice (*O. sativa*), and corn (*Z. mays*) under laboratory, greenhouse, and field conditions (Khan and others 2003; Prithviraj and others 2003). With respect to thuricin 17, Lee and others (2009) found that it can enhance soybean and corn growth. In keeping with those previous findings, Phase 1 showed that 10^{-6} M LCO treatment encouraged the early germination of *B. napus* cv. Polo, under standard temperature conditions, and Phase 2 showed that 10^{-6} M LCO treatment encouraged Topas germination at 10 and 25 °C.

Whittington (1973) concluded that maternal genetic factors strongly influenced germination rate in *B. napus*. Thus, 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. 2), is a clear example of the unintended and problematic effects of breeding for higher seed oil contents. 04C204's sister cultivar, 04C111, performed relatively well under all conditions tested here. This difference, and the difference in the responses of 02C3 and 02C6 to the respective signal molecules, despite the fact that they were produced by the same crosses of double haploid lines, may be deduced to be the product of genomic change during their generations of open pollination, or epigenetic changes. Changes to germination have been observed in other cultivars with altered seed oil contents (Linder 1998). Similarly, under continuous 20 °C temperature conditions, 04C111 germinated 212.1 % more, based on the inverse-linked germination count means, 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 continuous 30 °C temperature conditions (Fig. 4).

Subramanian (2014) 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, for example, *B. napus* germinated more rapidly under low

temperature, saline, and water-stressed conditions when pre-hydrated and re-dried, or when primed with ABA (Zheng and others 1998; Gao and others 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 and others 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 and others 2004; Wang and others 2011). 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.

Conclusions

The occurrence of potential microbe-to-plant signal-based communication was verified in the experimental cultivars, as the signal molecules elicited positive seed germination responses. The effects of new potential plant growth regulators, which have been shown previously to affect the germination of a variety of plants, were investigated using *B. napus* seeds with high and low (that is, Topas) oil concentrations, because oil quality has been shown to affect *B. napus* germination (Linder 1998). In the 5–15 GDD range of the early germination period, 75 % more Polo seeds germinated in 10^{-6} M LCO solution than in distilled water (Fig. 3). Further experimentation with other cultivars and other temperature conditions discerned a particular effect on Topas, following treatment with LCO, under abiotic stress (10 °C) and ideal (25 °C) growing conditions. The effect of LCO was specific to AOSA standard conditions, for the Polo cultivar, for which the effect of the 10^{-9} M signal had a stronger effect on the uniformity of germination than on the speed of germination. The effect of 10^{-6} M LCO was only discernable under abiotic stress and ideal temperature conditions for Topas.

Acknowledgments This work was completed thanks to funding acquired by Donald Smith from the Green Crop Network and the Eastern Canadian Oilseed Alliance.

Conflict of interest The authors declare that they have no conflict of interest.

References

Achard P, Gong F, Cheminant S, Alioua M, Hedden P, Genschik P (2008) The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. Plant Cell 20:2117–2129

Acharya SN, Dueck J, Downey RK (1983) Selection and heritability studies on canola/rapeseed for low temperature germination. Can J Plant Sci 63:377–384

Alberta Agriculture and Development (2012) Soil temperature and direct seeding http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/agdex1164. Accessed 12 Nov 2012

Association of Official Seed Analysts (1993) Rules for testing seeds. J Seed Technol 16(3):112

Atlas RM (1995) Handbook of media for environmental microbiology. CRC Press, Boca Raton

Bai Y, Souleimanov A, Smith DL (2002) An inducible activator produced by *Serratia proteamaculans* strain and its soybean growth-promoting activity under greenhouse conditions. J Exp Bot 53:1495–1502

Brown PD, Morra MJ (1996) Hydrolysis products of glucosinolates in *Brassica napus* tissues as inhibitors of seed germination. Plant Soil 181:307–316

Cameron RK, Paiva NL, Lamb CJ, Dixon RA (1999) Accumulation of salicylic acid and *PR-1* gene transcripts in relation to the systemic acquired resistance (SAR) response induced by *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*. Physiol Mol Plant Pathol 55:121–130

Carlson RW, Sanjuan J, Bhat R, Glushka J, Spaink HP, Wijfjes AHM, van Brussel AAN, Stokkermans TJW, Peters NK, Stacey G (1993) The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type I and II strains of *Bradyrhizobium japonicum*. J Biol Chem 268(24):18372–18381

Chen L, Zhong H, Ren F, Guo Q-Q, Hu X-P, Li X-B (2011) A novel cold-regulated gene, *COR25*, of *Brassica napus* is involved in plant response and tolerance to cold stress. Plant Cell Rep 30:463–471

Coffman FA (1923) The minimum temperature of germination of seeds. J Am Soc Agron 15(7):257–270

Dashti N, Zhang F, Hynes R, Smith DL (1997) Application of plant growth-promoting rhizobacteria to soybean (*Glycine max* [L.] Merr.) increases protein and dry matter yield under short-season conditions. Plant Soil 188:33–41

Doherty CJ, Buskirk H, Myers SJ, Thomashow MF (2009) Roles for Arabidopsis CAMTA transcription factors in cold: regulated gene expression and freezing tolerance. Plant Cell 21:972–984

Dunfield K, Srivastava S, Shah S, Kav NNV (2007) Constitutive expression of *ABR17* cDNA enhances germination and promotes early flowering in *Brassica napus*. Plant Sci 173:521–532

Dusenbery DB (1992) Sensory ecology: how organisms acquire and respond to information. W H Freeman Limited, New York 558 p

Elliot AC, Hynan LS (2010) Kruskal Wallis analysis with multiple comparisons. http://www.alanelliott.com/kw. Accessed 13 Feb 2013

Farhoudi R, Hussain M, Lee D-J (2012) Modulation of enzymatic antioxidants improves the salinity resistance in canola (*Brassica napus*). Int J Agric Biol 14:465–468

Felix G, Regenass M, Boller T (1993) Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state. Plant J 4:307–316

Gao Y-P, Bonhma-Smith PC, Gusta LV (2002) The role of peroxiredoxin antioxidant and calmodulin in ABA-primed seeds of *Brassica napus* exposed to abiotic stresses during germination. J Plant Physiol 159:951–958

Genre A, Chabaud M, Balzergue C, Puech-Pagès V, Novero M, Rey T, Fournier J, Rochange S, Bécard G, Bonfante P, Barker DG (2013) Short-chain chitin oligomers from arbuscular mycorrhizal

fungi trigger nuclear Ca^{2+} spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. New Phytol 198:179–189

Giavalisco P, Kapitza K, Kolasa A, Buhtz A, Kehr J (2006) Toward the proteome of *Brassica napus* phloem sap. Proteomics 6: 896–909

Goedhart J, Hink MA, Visser AJWG (2000) In vivo fluorescence correlation microscopy (FCM) reveals accumulation and immobilization of Nod factors in root hair cell walls. Plant J 21(1): 109–119

Gough C, Cullimore J (2011) Lipo-chitooligosaccharide signaling in endosymbiotic plant-microbe interactions. Mol Plant Microbe Interact 24(8):867–878

Gray EJ, Di Falco M, Souleimanov A, Smith DL (2006) Proteomic analysis of the bacteriocin thuricin 17 produced by *Bacillus thuringiensis* NEB17. FEMS Microbiol Lett 255:27–32

Groves P, Offermann S, Rasmussen MO, Cañada FJ, Bono JJ, Driguez H, Imberty A, Jiménez-Barbero J (2005) The relative orientation of the lipid and carbohydrate moieties of lipochitooligosaccharides related to nodulation factors depends on lipid chain saturation. Org Biomol Chem 3:1381–1386

Guan Y-J, Hu J, Wang X-J, Shao C-X (2009) Seed priming with chitosan improves maize germination and seedling growth in relation to physiological changes under low temperature stress. J Zhejiang Univ Sci B 10(6):427–433

Hamel LP, Beaudoin N (2010) Chitooligosaccharide sensing and downstream signaling: contrasted outcomes in pathogenic and beneficial plant–smicrobe interactions. Planta 232:787–806

Harker KN, Clayton GW, Blackshaw RE, O'Donovan JT, Stevenson FC (2003) Seeding rate, herbicide timing and competitive hybrids contribute to integrated weed management in canola (*Brassica napus*). Can J Plant Sci 83:433–440

Harker KN, O'Donovan JT, Blackshaw RE, Johnson EN, Lafond GP, May WE (2012) Seed depth and seeding speed effects on no-till canola emergence, maturity, yield and seed quality. Can J Plant Sci 92:795–802

Jung W-J, Mabood F, Souleimanov A, Zhou X, Jaoua S, Kamoun F, Smith DL (2008) Stability and antibacterial activity of bacteriocins produced by *Bacillus thuringiensis* and *Bacillus thuringiensis* ssp. k*urstaki*. J Microbiol Biotechnol 18(11):1836–1840

Jung W-J, Mabood F, Souleimanov A, Smith DL (2011) Induction of defense-related enzymes in soybean leaves by class IId bacteriocins (thuricin 17 and bacthuricin F4) purified from *Bacillus* strains. Microbiol Res 167:14–19

Khan W, Prithiviraj B, Smith DL (2003) Chitosan and chitin oligomers increase phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities in soybean leaves. J Plant Physiol 160:859–863

Kucera B, Cohn MA, Leubner-Metzger G (2005) Plant hormone interactions during seed dormancy release and germination. Seed Sci Res 15:281–307

Lee KD, Gray EJ, Mabood F, Jung W-J, Charles T, Clark SRD, Ly A, Souleimanov A, Zhou X, Smith DL (2009) The class IId bacteriocin thuricin-17 increases plant growth. Planta 229: 747–755

Liang Y, Cao Y, Tanaka K, Thibivilliers S, Wan J, Choi J, Kang Ch, Qiu J, Stacey G (2013) Nonlegumes respond to rhizobial Nod factors by suppressing the innate immune response. Science 341:1384–1387

Linder CR (1998) Potential persistence of transgenes: seed performance of transgenic canola and wild × canola hybrids. Ecol Appl 8(4):1180–1195

Marshall B, Squire GR (1996) Non-linearity in rate-temperature relations of germination in oilseed rape. J Exp Bot 47(302):1369–1375

Médiène S, Valantin-Morison M, Sarthou J-P, de Tourdonnet S, Gosme M, Bertrand M, Roger-Estrade J, Aubertot J-N, Rusch A, Motisi N, Pelosi C, Doré T (2011) Agroecosystem management and biotic interactions: a review. Agron. Sustain. Dev. 31: 491–514

Morando MA, Nurisso A, Grenouillat N, Vauzielles B, Beau J-M, Cañada FJ, Jiménez-Barbero J, Imberty A (2011) NMR and molecular modeling reveal key structural features of synthetic nodulation factors. Glycobiology 21(6):824–833

Nykiforuk CL, Johnson-Flanagan AM (1999) Storage reserve mobilization during low temperature germination and early seedling growth in *Brassica napus*. Plant Physiol Bioch 37(12):939–947

Ohnuma T, Onaga S, Murata K, Taira T, Katoh E (2008) LysM domains from *Pteris ryukyuensis* chitinase-A: a stability study and characterization of the chitin-binding site. J Biol Chem 283(8):5178–5187

Ohta K, Atarashi H, Shimatani Y, Matsumoto S, Asao T, Hosoki T (2000) Effects of chitosan with or without nitrogen treatments on seedling growth in *Eustoma grandiflorum* (Raf.) Shinn. Cv. Kairyou Wakamurasaki. J Japan Soc Hort Sci 69(1):63–65

Oldroyd GE, Murray JD, Poole PS, Downie JA (2010) The rules of engagement in the legume-rhizobial symbiosis. Ann Rev Genet 45:119–144

Omidi H, Tahmasebi Z, Badi HAN, Torabi H, Miranseri M (2010) Fatty acid composition of canola (*Brassica napus* L.), as affected by agronomical, genotypic and environmental parameters. C R Biol 333:248–254

Op den Camp R, Streng A, De Mita S, Cao Q, Polone E, Liu W, Ammiraju JSS, Kudrna D, Wing R, Untergasser A, Bisseling T, Geurts R (2011) LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume *Parasponia*. Science 331: 909–912

Petutschnig EK, Jones AME, Serazetdinova L, Lipka U, Lipka V (2010) The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-inuced phosphorylation. J Biol Chem 285: 28902–28911

Piegorsch WW, Bailer AJ (2005) Analyzing environmental data. Wiley, Chichester

Prithiviraj B, Zhou X, Souleimanov A, Kahn WM, Smith DL (2003) A host-specific bacteria-to-plant signal molecule (Nod factor) enhances germination and early growth of diverse crop plants. Planta 216:437–445

Rinaudo M (2006) Chitin and chitosan: properties and applications. Prog Polym Sci 31:603–632

Sangwan V, Foulds I, Singh J, Dhindsa RS (2001) Cold-activation of *Brassica napus* BN115 promoter is mediated by structural changes in membranes and cytoskeleton, and requires Ca^{2+} influx. Plant J 27:1–12

Sanjuan J, Carlson RW, Spaink HP, Bhat UR, Barbour WM, Glushka J, Stacey G (1992) A 2-*O*-methylfucose moiety is present in the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*. Proc Natl Acad Sci USA 89:8789–8793

Savitch LV, Allard G, Seki M, Robert LS, Tinker NA, Hüner NPA, Shinozaki K, Singh J (2005) The effect of over-expression of two Brassica CBF/DREB1-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus*. Plant Cell Physiol 46:1525–1539

Saxton AM (1998) A macro for converting mean separation output to letter groupings in Proc Mixed. In: Proceedings of 23rd SAS Users Group Intl., SAS Institute, Cary, pp 1243–1246

Schielzeth H, Nakagawa S (2013) Nested by design: model fitting and interpretation in a mixed model era. Method Ecol Evol 4:14–24

Shaykewich CF (1973) Proposed method for measuring swelling pressure of seeds prior to germination. J Exp Bot 24(83): 1056–1061

Silvertown J (1980) Leaf-canopy-induced dormancy in a grassland flora. New Phytol 85:109–118

Sit V, Poulin-Costello M (1994) Catalogue of curves for curve fitting. Forest Science Research Branch, Victoria

Souleimanov A, Prithiviraj B, Carlson RW, Jeyaretnam B, Smith DL (2002a) Isolation and characterization of the major nod factor of *Bradyrhizobium japonicum* strain 532C. Microbiol Res 157: 25–28

Souleimanov A, Prithiviraj B, Smith DL (2002b) The major Nod factor of *Bradyrhizobium japonicum* promotes early growth of soybean and corn. J Exp Bot 53(376):1929–1934

Srivastava S, Fristensky B, Kav NNV (2004) Constitutive expression of a PR10 protein enhances the germination of *Brassica napus* under saline conditions. Plant Cell Physiol 45(9):1320–1324

Staehelin C, Granado J, Muller J, Wiemken A, Mellor RB, Felix G, Regenass M, Broughton WJ, Boller T (1994) Perception of *Rhizobium* nodulation factors by tomato cells and inactivation by root chitinases. Proc Natl Acad Sci USA 91:2196–2200

Subramanian S (2014) Mass spectrometry based proteome profiling to understand the effects of lipo-chito-oligosaccharide and thuricin 17 in *Arabidopsis thaliana* and *Glycine max* under salt stress. Ph.D. Thesis. McGill University, Montreal

Wan J, Zhang X-C, Neece D, Ramonell KM, Clough S, Kim S-Y, Stacey MG, Stacey G (2008) A LysM receptor-like kinase plays a critical role in chitin signalling and fungal resistance in *Arabidopsis*. Plant Cell 20:471–481

Wang S-L, Hsu W-H, Liang T-W (2010) Conversion of squid pen by *Pseudomonas aeruginosa* K187 fermentation for the production of *N*-acetyl chitooligosaccharides and biofertilizers. Carbohyr Res 345:880–885

Wang J-M, Fan ZY, Z-b Liu, Xiang J-B, Chai L, Li X-F, Yang Y (2011) Thylakoid-bound ascorbate peroxidase increases resistance to salt stress and drought in *Brassica napus*. Afr J Biotechnol 10(41):8039–8045

Whittington WJ (1973) Genetic regulation of germination. In: Heydecker W (ed) Seed ecology. Proceedings of the 19th Easter School in Agricultural Science. University of Nottingham, Butterworth

Yao K, Lockhart KM, Kalanack JJ (2005) Cloning of dehydrin coding sequences from *Brassica juncea* and *Brassica napus* and their low temperature-inducible expression in germinating seeds. Plant Physiol Bioch 43:83–89

Zheng GH, Gao YP, Wilen RW, Gusta LV (1998) Canola seed germination and seedling emergence from pre-hydrated and re-dried seeds subjected to salt and water stresses at low temperatures. Ann Appl Biol 132:339–348

Zhuang J, Sun C-C, Zhou X-R, Xiong A-S, Zhang J (2011) Isolation and characterization of an AP2/ERF-RAV transcription factor BnaRAV-1-HY15 in *Brassica napus* L. HuYou15. Mol Biol Rep 38:3921–3928