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Rhizobial Lipo-Chitooligosaccharides and Gibberellins Enhance Barley (*Hordeum vulgare* L.) Seed Germination

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Abstract: Gibberellins are plant hormones, enhancing seed germination. The bacterium-to-plant signal, lipo-chitooligosaccharides (LCOs) or Nod factors, are of great importance for roots organogenesis and hence, nodule formation and N fixation. Hence, we hypothesized that LCOs like gibberellins may also enhance barley (*Hordeum vulgare* L.) germination. The objectives were to test the effects of gibberellins on barley germination and to test the hypothesis that LCOs may increase seed germination in barley. The concentrations, tested were 10^{-5} M for gibberellins and 10^{-6} M and 10^{-7} and 10^{-8} M LCOs. Although, gibberellins were able to numerically increase barley germination (up to 18%), the LCOs seemed to be more effective on barley germination as they significantly increased seed germination (up to 44%). Hence, the novel finding indicates that for LCOs may also be very effective on barley seed germination, through inducing morphogenesis and physiological changes in seeds. This finding can have very important agricultural implications.

Key words: Barley (*Hordeum vulgare* L.) seed germination, *Bradyrhizobium japonicum* signal, lipo-chitooligosaccharides, nodule formation, root morphogenesis

INTRODUCTION

The process of symbiosis between *Bradyrhizobium japonicum* and soybean (*Glycine max* L. Merr.) begins with the excretion of the plant-to-bacterium signal, genistein, which is a bacterial *nod* gene inducer (Miransari and Smith, 2007, 2008), followed by the production of the rhizobia-to-plant signal, lipo-chitooligosaccharides (LCOs), or Nod factors (Bai *et al*., 2002). Lipo-chitooligosaccharides are those molecules, responsible for morphogenesis changes in the roots and hence, nodule formation (Miransari *et al*., 2006; Khan *et al*., 2008).

The combination of the oligosaccharides of β-1,4-linked N-acetyl-o-glucosamine and of some functional groups form LCOs. According to Perret *et al*. (2000) the production of LCOs is related to some complicated biochemical processes taking place with the help of enzymes, produced and activated as a result of *nod* genes expression. Different strains of rhizobium produce various mixtures of LCO and the strain, *Bradyrhizobium japonicum* 532C, produces nod Bj V ($C_{18:1}$; MeFuc) (Prithiviraj *et al*., 2000; Khan *et al*., 2008).

Submicromolar concentrations of LCOs may result in physiological changes in legumes (Goedhart *et al*., 2003) and non-legumes (Souleimanov *et al*., 2002; Prithiviraj *et al*., 2003). Although, it is not clear yet how plants perceive LCOs (Macchiavelli and Brelles-Marino, 2004), it has been suggested that there are more than one LCO perception system (Cullimore *et al*., 2001) including two different LCO binding sites for *Medicago* sp. called NFBS1 and NFBS2, with different affinities (Gressent *et al*., 1999).

The LCOs are able to form (Macchiavelli and Brelles-Marino, 2004) and deform root hairs (Miransari *et al*., 2006), depolarize membrane, alkalinize the internal and external cellular space, induce the expression of *nod* genes, alternate ion fluxes and form nodules (Broughton *et al*., 2000). It has also been reported that LCOs may enhance seed germination in some crops (Prithiviraj *et al*., 2003), though there has not yet been any report on the enhancing effect of LCOs on seed germination in barley.

Macchiavelli and Brelles-Marino (2004) have stated that treating seeds of *Medicago trancatula* with LCOs may more effectively increase the number of nodules than when roots are not treated and have attributed this to the existence of a perception system with a high affinity for LCO in the seed and embryo of *M. trancatula*.

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Seeds of cereals include endosperm providing the necessary nutrients, carbohydrates and proteins for seed growth before the beginning of seedling photosynthesis. It is made of two parts, the central starchy part and the surrounding aleurone (Bosnes *et al*., 1992). The barley (*Hordeum vulgare*) endosperm becomes available to the embryo as a result of hydrolases production during germination (Jones and Jacobsen, 1991).

While, the plant hormone, gibberellins, stimulates the synthesis and excretion of these enzymes (mainly α-amylases) and hence seed germination, abscisic acid (ABA) adversely affects these processes (Jones and Jacobsen, 1991). Hence, barley aleurone is of great significance for the study of signal transduction pathways in response to the plant hormones, gibberellins and ABA and some of these pathways have yet to be elucidated (Ritchie and Gilroy, 1998).

Gibberellins are involved in the induction of several genes, necessary for the synthesis and secretion of α-amylase before germination. The embryo synthesizes gibberellins, residing in the aleurone (Appleford and Lenton, 1997), where they are programmed for the expression of genes, responsible for the synthesis and secretion of α-amylases, proteases and β-glucanases.

Although, there are findings on the effects of exogenously applied gibberellins on barley seed germination, but since to our knowledge there are not any findings on the effects of LCO on barley seed germination, we hypothesized that with respect to their properties, LCOs are also able to induce and enhance seed germination in barley. We proposed these experiments to further exhibit the great importance of microbiological products in the stability and production of ecosystems and indicate that legumes can also be very useful to non-legumes per se in a intercropping or rotational cropping. The objectives were (1) to test the effects of gibberellins on barley seed germination and (2) to test the hypothesis that LCO may increase seed germination in barley, though it may be concentration dependent.

MATERIALS AND METHODS

Two experiments were conducted in which the following stages were taken. Seeds of barley (*Hordum vulgare* L.) were surface sterilized in sodium hypochlorite (2% solution containing 4 ml L^{-1} Tween 20) and rinsed several times with distilled water (Bhuvaneswari *et al*., 1980). There were eight seeds per treatment in the first experiment and to increase the experimental precision 20 seeds per treatment in the second experiment. In both experiments seeds were grown in Petri dishes in the laboratory, at room temperature, with 16 h of fluorescence light in the month of October at the laboratory of Plant Science Department, Macdonald College of McGill University, Montreal, Canada.

In the first experiment distilled water was used as the control treatment and concentrations of 10^{-5} and 10^{-6} M of gibberellins (Sigma-Aldrich Canada Ltd., Oakville, Canada) and LCO (produced, isolated and purified as explained below) were applied to the seeds in Petri dishes (9 for each treatment) containing filter papers and 5 mL of the solution (Benech-Arnold *et al*., 2003).

In the second experiment also similar stages were taken but in addition to the treatments, used in the first experiment, concentrations of 10^{-7} and 10^{-8} M of LCO were also applied to ten Petri dishes for each treatment. Twenty hours after applying the treatments the seed germination rates were determined.

Production, extraction and purification of LCO: LCOs were produced, extracted and purified taking the following stages (Miransari *et al*., 2006).

Bacterial culture: *Bradyrhizobium japonicum* strain 532C was grown at 28°C in 100-125 mL of sterile yeast mannitol medium (YEM) (pH 6.8) containing 10 g mannitol, 0.5 g K_2HPO_4, 0.0977 g $MgSO_4$, 0.1 g NaCl, 0.4 g yeast extract and 1000 mL distilled water. The culture was shaken at 150 rpm until it achieved an OD_{620} equal to 0.4-0.6 (4-6 days).

Thereafter, 2 L bacterial subculture was prepared by adding 5 mL of the culture to 250 mL of YEM medium and 0.25 mL of 50 μM genistein was added to each 250 mL of bacterial subculture. The subculture was grown at 28°C and shaken at 150 rpm until the OD_{620}, reached 0.8-1.0 (5-7 days). Concentration of 5 μM genistein in the subculture was produced, while looking white-grayish. The LCO biosynthesis is induced by genistein over the course of 48-96 h.

Lipo-chitooligosaccharide extraction: To the bacterial subculture 0.8 L of HPLC grade 1-butanol was added and it was shaken for 5 min and then left over night to separate into organic and water phases. Using a separatory funnel the 2 layers were carefully separated. The lower layer was discarded and the upper one was collected and stored at 4°C before it was concentrated by evaporation. A rotary evaporator (Yamato RE500) and temperature of 80°C were used to evaporate the collected layer. The remaining material was dissolved in 4 mL of 18% acetonitrile and kept in the dark in a glass vial sealed with parafilm.

Purification of LCO by HPLC: For the HPLC analysis a Vydac C18 reversed-phase column (0.46×25 cm; 5 μM) with a flow rate of 1.0 mL min^{-1} and a Vydac guard column were used. To establish a baseline, 18% acetonitrile (AcN/H_2O; w/w) was used and this baseline was run for at least 10 min. The baseline should be about 0.01. If the injection volume was greater than 200 μL, the auxiliary loop was installed for Waters 712 WISP Autosampler. After injecting the sample, isocratic elution by 18% of AcN for 45 min was started. This step removes all non-polar junk light fractions. Thereafter, gradient elution for 90 min was applied using 18-82% of AcN. LCO should begin to appear at the detector at 94-96 min after starting the run (identified by using a standard) (Souleimanov *et al*., 2002). When the water fraction collector (collects a new fraction every 2 min) was used, LCO was found in fractions, numbered 47 and 48.

Statistical analysis: Using the SAS (1988) system the analysis of variance was performed for the data and the effect of the model and the main effect in the model, treatments, were statistically determined. Mean values were compared using the GLM method and the least significant difference test at p = 0.05 (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

In the first experiment, adding LCO at 10^{-6} M increased seed germination (by 44%) significantly, compared to the control, while adding gibberellins at 10^{-5} M did not. Both the effects of model and treatment were significant (p = 0.05) (Table 1).

In the second experiment, the effects of both the model and the treatment were also significant. In the second experiment, adding LCO at 10^{-7} M increased seed germination (by 25%) significantly compared to the control while adding gibberellins at 10^{-5} M or LCO at either 10^{-6} or 10^{-8} M did not. The effect of LCO 10^{-8} M was not different from control (Table 1).

Table 1: The effects of different concentrations of LCO and gibberellins on barley seed germination (±SD) in the first and second experiment

Treatments	Seed germination (%)
First experiment	
Control	62.5±13.2b
Gibberellins 5	73.6±10.7b
LCO6	90.3±9.6a
Model	**
Treatment	**
LSD	14.1
Second experiment	
Control	48.5±8.55b
Gibberellins 5	53.0±8.1ab
LCO6	58.0±13.9ab
LCO7	60.5±12.4a
LCO8	48.5±9.7b
Model	*
Treatment	*
LSD	9.7

Gibberellins 5: Gibberellins at 10^{-5} M; LCO6, 7 and 8: LCO at 10^{-6}, 10^{-7} and 10^{-8} M, respectively. LSD: Least significant difference test. Values within the same column, followed by different letter(s) are significantly different at p = 0.05; * = p<0.05; ** = p<0.01

The effects of LCOs on barley germination: Although, the key role of LCOs in nodule formation has been determined, there are other plant morphogenesis activities, attributed to LCOs including the stimulation of genes, involved in cell cycling in the cultures of suspension cell and stimulation of mitosis division in the cultures of protoplast in both legumes and non-legumes (Souleimanov *et al*., 2002) and also Ca^{2+} spiking in the cytoplasm (Mitra *et al*., 2004; Kalo *et al*., 2005). After 1 min of addition, Nod factors can depolarize the membrane of legume root hairs (Ehrhardt *et al*., 1992; Miwa *et al*., 2006), as a result of Ca^{2+} spiking at the cytoplasm of root hair tip (Cardenas *et al*., 1998; Shaw and Long, 2003).

Present results show that LCOs are capable of enhancing barley seed germination, which is in agreement with Bai *et al*. (2002), who indicated that LCOs might induce seed germination in soybean. In the soil, LCOs may have many enhancing effects on plant growth through inducing seed germination (Zhang and Smith, 2001), enhancing plant growth, development and yield for both legumes and non-legumes, increasing the photosynthetic rates when sprayed onto the leaves (Smith *et al*., 2002; Almaraz *et al*., 2006), stimulating effects on flavonoid genes (Spaink and Lugtenberg, 1994), enhancing the ability of arbuscular mycorrhiza in root colonization (Xie *et al*., 1995) and cell cycling and embryogenesis. In addition LCO can also later the auxin transfer (Souleimanov *et al*., 2002), which its balance with cytokinin can affect plant growth (Relic *et al*., 1993). Although, it has yet to be specified whether there are LCOs receptors on the seed and embryos, however scientists have suggested that there are LCOs receptors on the roots of developing embryos (Macchiavelli and Brelles-Marino, 2004).

Based on present results, LCO 10^{-7} M may be the most effective concentration to induce seed germination in barley, which is in accordance with Zhang and Smith (2001), Smith *et al*. (2002) and Supanjani *et al*. (2005). This stimulating effect is also in agreement with the idea that LCOs are capable of adjusting plant growth through restoration and continuation of cell cycling and embryogenesis in mutated plants and cultures of somatic embryo (De Jong *et al*., 1993; Egertsdotter and Von Arnold, 1998) when the plant hormones, auxins and cytokinins are not present (Daychok *et al*., 2000).

Also, foliar application of LCOs at 10^{-6}, 10^{-8} and 10^{-10} M onto the leaves increased the photosynthetic rates from 10 to 20% in soybean, common bean, maize (Khan *et al*., 2008), rice, canola, apple and grape plants. This enhanced photosynthetic rate resulted in an increase of 40% in soybean yield under field conditions (Smith *et al*., 2002). LCOs in rhizobia may be overexpressed by phenolic compounds, which are abundant in plant root exudates and residues (Dakora, 2003). When presented at high concentrations in the soil, these signal molecules may result in the production of large amounts of LCOs in the rhizosphere, with high inducing effects on plant growth (Dakora, 2003).

Gibberellins and barley germination: Present results show that exogenously applied gibberellins enhanced the germination of barley seeds, which is in agreement with Steinbach *et al*. (1997) and Benech-Arnold *et al*. (2000), who stated that exogenous gibberellins increased germination in developing seeds. Also, application of gibberellins may enhance seed germination in dormant seeds as the biosynthesis of gibberellins is suppressed in dormant seeds compared with non-dormant seeds (Pérez-Flores *et al*., 2003). The balance between gibberellins and ABA is a determining factor in the germination capacity of seeds (Benech-Arnold *et al*., 2003).

To prove the hypothesis that gibberellins are necessary for the germination of immature embryo, White and Rivin (2000) applied paclobutrazol and ancymidol, the inhibitors of gibberellins synthesis, to the cultured embryo at different stages of development. The dissection of embryos took place at different stages and also the ratio and the part of the embryos, germinated contingently decreased. Addition of exogenous gibberellins could suppress the effects of gibberellins inhibitors enabling embryos to germinate. Also, when gibberellins inhibitors are not present, exogenous gibberellins could significantly enhance embryos germination.

CONCLUSION

With regard to the results of these experiments it is very much clear that LCOs are able to enhance barley seed germination even more effectively than gibberellins through morphogenesis and physiological changes. This can indicate the great importance of biological (i.e., secondary metabolites) products in enhancing barley seed germinating and growth. Although, barely is a non-legume plant, however the legume LCOs products in a rotation or intercropping (Prithiviraj *et al*., 2003; Khan *et al*., 2008) can very much contribute to the increased level of productivity (Long, 2005). It should also be mentioned that to our knowledge data regarding the effects of LCOs and barley germination are rare.

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