

Effect of Lipo-chitooligosaccharide on Germination and Seedling Growth of Cauliflower

Peranan Lipo-kitooligosakarida pada Perkecambahan dan Pertumbuhan Awal Kubis Bunga

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ABSTRACT

Lipo-chitooligosaccharide (LCO) has been shown to induce cell division and somatic embryo development in carrot and Norway Spruce cells. Experiments were conducted to test the effect of NodBj-V(C18:1,Mefuc), the main LCO being produced by genistein-induced-*B. japonicum* 532C, on cauliflower seed germination and seedling growth under controlled conditions in Petri plates. LCO was used at concentrations of 10^{-8} M and 10^{-10} M. LCOs increased germination speed (T_{50}) 8 hours from 58 hours, increased 69% of total root length, and increased about 30% of total surface/projected area of the roots. Leaf area, hypocotyl length and seedling weights were not affected by LCO treatment. The possible role of Ca^{2+} as a second messenger in signal transduction for growth induction of LCO was explored using standard pharmacological agents, Verapamil (a plasmamembrane calcium channel blocker) and chlorpromazine (a Calmodulin antagonist). An addition of verapamil or chlorpromazine to the LCO's medium nullified the positive effects of LCO, suggesting the role of Ca^{2+} in the signal transduction of LCO activity. Interestingly, excessive amount of Ca^{2+} in the medium overcame the inhibitory effect of verapamil.

Key words: Lipo-chitooligosaccharide, seed germination, verapamil, chlorpromazine

ABSTRAK

Lipo-kitooligosakarida (LCO) telah terbukti menginduksi pembelahan sel dan perkembangan embrio somatic pada sel Norway Spruce. Penelitian dilaksanakan untuk menguji pengaruh NodBj-V(C18:1,Mefuc), LCO utama yang diproduksi oleh *B. japonicum* 532C yang diinduksi dengan genistein, dievaluasi pengaruhnya terhadap perkecambahan dan pertumbuhan kecambah benih kubis bunga yang diinkubasi di cawan petri di dalam cabinet pertumbuhan. LCO digunakan pada konsentrasi 10^{-8} M and 10^{-10} M. LCO meningkatkan kecepatan perkecambahan (T_{50}), total panjang akar, dan total permukaan/luas proyeksi akar. Namun demikian, luas daun, panjang hipokotil, dan berat kecambah tidak dipengaruhi oleh perlakuan LCO. Pengaruh LCO kemungkinan melibatkan Ca^{2+} sebagai perantara kedua dieksplorasi dengan menggunakan agensia standar penghambat aktivitas kalsium, yaitu Verapamil (penghambat saluran kalsium di plasmamembran) dan klorpromazin (antagonis dari kalmodulin). Penambahan verapamil atau klorpromazin ke dalam medium yang mengandung LCO menghilangkan pengaruh positif dari LCO, memperlihatkan bahwa Ca^{2+} berperan dalam transduksi sinyal dari aktivitas LCO. Hal ini juga diperkuat bahwa penambahan Ca^{2+} yang berlebih dalam medium dapat mengatasi pengaruh penghambatan pertumbuhan oleh verapamil.

Kata kunci: lipo-kitooligosakarida, perkecambahan, verapamil, klorpromazin

INTRODUCTION

The legume-rhizobia symbiosis is a complex interaction between higher plants and prokaryotes, and this requires the interplay of at least two sets of signal molecules. The first is the plant-to-bacteria signals which are usually flavonoids and isoflavonoids found in the root exudates and the second is the bacteria-to-plant signals, lipo-chitoooligosaccharides (LCOs) or nod factors (Long, 1989; Kondorosi, 1991; Boone *et al.*, 1999). In general, LCOs are composed of three to five 1-4 β -linked acetyl glucosamine residues with the N acetyl group of the terminal non-reducing end replaced by an alkyl chain. However, various modifications to this basic structure are possible and each of the rhizobia produces a specific set of LCOs. Some studies have implicated LCOs as a possible candidate in the host specificity of rhizobia (Spaink *et al.*, 1991; Schultze *et al.*, 1992; Cohn-*et al.*, 1998; Stougaard, 2000). LCOs are known to affect a number of host plant physiological processes, such as inducing root hair deformation (Spaink *et al.*, 1991) ontogeny of compete nodule structures (Fisher and Long, 1992; Denarie and Cullimore, 1993), cortical cell division (Sanjuan *et al.*, 1992; Schulaman *et al.*, 1997) and the expression of host nodulin genes essential for the infection thread formation (Horvath *et al.*, 1993; Minami *et al.*, 1996). LCOs have also been shown to activate defense-related enzymes (Inui *et al.*, 1997).

In non-host plants, LCOs have been shown to elicit several physiological responses. Applications of LCOs induces rapid and transient alkalination of tobacco (Baier *et al.*, 1999) and tomato cells (Stahlein *et al.*, 1994) in suspension cultures. De Jong *et al.* (1993) found that LCOs restored cell division and embryo development in temperature-sensitive carrot mutant. Somatic embryo cultures of Norway spruce (*Picea abies*) also resumes cell division when LCOs were added to the medium (Egertsdotter and von Arnold, 1998), even in the absence of auxin and cytokinin (Daychok *et al.*, 2000). In addition, Supanjani *et al.* (2006) found that LCO produced by *Bradyrhizobium japonicum* increase Ca^{2+} uptake from root medium of soybean plant. However, the effect of LCOs on whole plant

system of non-host plants has not been studied.

The role of Ca^{2+} as a second messenger in higher plants has become clear during the last two decades (Hepler and Wayne, 1985). In plants, it is responsible for invoking physiological responses to various cues like gravitropism (Daye *et al.*, 1984) and anoxia (Subbaiah *et al.*, 1994). The role of Ca^{2+} in plants has been deciphered by the use of various pharmacological agents such as verapamil and chlorpromazine (Hepler and Wayne 1985). Ca^{2+} has been shown to be involved in the signal transduction leading to LCO's effects. Calcium sensitive fluorescent dye has revealed that nod factor caused a transient increase in intracellular calcium in nodulating alfalfa but not in a non-nodulating mutant (Ehrhardt *et al.*, 1996). It has been suggested that Ca^{2+} plays a primary role in the transduction of the LCO signal (Felle *et al.* 1998) in a host-specific manner (Yokoyama *et al.*, 2000). Physiological responses in host plant roots include rapid (within seconds) transient increases in cytosolic Ca^{2+} influx (Allen *et al.*, 1994, Felle *et al.*, 1998) both from intercellular space and from the vacuole (Felle *et al.*, 1999). Felle *et al.* (1998) reported that the Ca^{2+} ionophore can mimic LCO's responses and the Ca^{2+} channel blocker nifedipine inhibits this response. Recently Webb *et al.* (2000) showed that LjCbp1, a gene encoding a putative Ca^{2+} binding protein, is expressed in a LCO dependent fashion in root epidermal cells.

Although a number of reports on the role of Ca^{2+} in physiological responses of host plants to LCO are in the literature there is a dearth of information of the role of Ca^{2+} in the physiological responses of non-host plants to LCO. The objective of the research was to determine the effect of LCO on the germination of cauliflower seed, and to determine whether this effect is mediated by Ca^{2+} by using verapamil, a plasma membrane Ca^{2+} channel blocker, and chlorpromazine, a calmodulin antagonist, in combination with LCO.

MATERIAL AND METHODS

Materials

Seeds of the cauliflower hybrid Chieftain

were a gift from Petoseeds Co. Inc (Saticoy, CA, USA) and were stored at 4°C until use. MS medium, verapamil and Chlorpromazine were purchased from Sigma Chemical Co. (St. Louis MO, USA). Gelrite (K9A40 Gellan gum) was purchased from Kelco (a division of Merck and Co. Inc, San Diego CA, USA).

Isolation and Purification of Lipo-Chitoooligosaccharide from *Bradyrhizobium japonicum* strain 532C

Bradyrhizobium japonicum (strain 532C) was grown at 28°C in yeast extract mannitol medium (YEM) (Mannitol 10g, K₂HPO₄ 0,5 g, MgSO₄ 7H₂O 0.2g, NaCl 0,1 g, yeast extract 0,4 g and distilled water 1000 mL), pH 6,8, shaken at 150 rpm until the OD₆₂₀ reached 0,4-0,6 (4-6 days) in the dark. Thereafter, 2 L of bacterial subculture was started by inoculating with material from the first culture (5 mL of the first culture per 250 mL of YEM media), for 5-7 days (OD₆₂₀ 0,8-1,0), as above. At this stage, 0.25mL of 50 µM genistein (in methanol) was added to each 250 mL of bacterial subculture (genistein concentration of 5 µM) and the culture was incubated for 48-96 hours.

Two litres of bacterial subculture were phase-partitioned against 0,8 L of HPLC-grade 1-butanol by shaking overnight. The upper butanol layer was transferred to an evaporation flask and concentrated to 2-3 mL of light brown, viscose material with a rotary evaporator operated at 80°C (Yamota RE500, Yamato, USA). This extract was resuspended in 4 mL of 18% acetonitrile and kept in the dark at 4 °C in a sealed glass vial until use.

HPLC analysis (Waters, MA, USA) was conducted with a Vydac C18 reversed-phase column (Vydac, CA, USA) with a flow rate 1.0 mL min⁻¹ and a Vydac guard column. As a baseline 18% acetonitrile in H₂O (w/w) was run through the system for at least 10 min prior to injection. The sample was loaded and isocratic elution was conducted with 18% acetonitrile for 45 min to remove all non-polar light fractions. Thereafter, gradient elution was conducted for 90 min. with 18-82% acetonitrile. The LCO eluted at 94-96 min of HPLC run time. The identity of the material was further confirmed by FAB-MS spectrometry (University of Georgia, USA).

Table 1. Effect of Lipo-chitoooligosaccharide (NodBj-V(C18:1,Mefuc)) on cauliflower seed germination and seedling growth

Treatment	T ₅₀ (h)	Leaf Area (cm ²)	Seedling Weight (mg)
Control	58,7 a	1,99	4,07 b
LCO 10 ⁻⁸ M	52,7 b	2,70	4,92 ab
LCO 10 ⁻¹⁰ M	50,7 b	2,66	5,12 a

Keterangan : Values within the same column followed by the same letter are not significantly different at P_{0.05}.

Effect of LCO on Seed Germination and Seedling Growth

Two concentrations of LCO were used (10⁻⁸ and 10⁻¹⁰ M). These two concentrations were selected based on a previous experiment in which a wide range of concentrations from 10⁻⁵ to 10⁻¹⁵ was tested for effects on the germination on several crop plants (unpublished results). Twenty ml of half strength MS medium (2,2 g L⁻¹) (Sigma Culture™, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), containing 1,45 mM Ca²⁺, amended with 15g L⁻¹ of sucrose containing the required concentration of LCO was dispensed into 9 cm diameter plastic Petri dishes (Fisher Scientific, Montreal, Canada). Gelrite was used as the gelling agent (2 g L⁻¹) because of its superior transparency as compared to standard agar. Seeds of cauliflower were surface-sterilized in 70% ethanol for 5 min, followed by 20 min in 10% Javex (commercial bleach containing 5.5% sodium hypochlorite) containing 0,05% Tween-20. Seeds were then washed with at least four times of sterile deionized water. Ten seeds were placed equidistantly in the Petri dishes. The plates were then sealed with parafilm (American National Can, Menasha, WI) and placed under two continuous fluorescent light bulbs (F40CWRS Watmiser, GE, Canada) at a constant temperature of 22 °C. Each treatment was replicated three times and the experiment was repeated twice.

Germination was observed at 6-hour intervals in order to allow determination of the time when 50% of the viable seeds had germinated (T₅₀). T₅₀ was calculated by the following formula (Coolbear *et al.*, 1984):

$$T_{50} = t_1 + [(G/2 - g_1)/(g_2 - g_1)](t_2 - t_1)$$

[where, t₁ and t₂ are time of observations before and after T₅₀, respectively; g₁ and g₂ are

observed total germination before and after T_{50} ; and G is the germination at the final observation]

Seedling growth was measured after 10 days of incubation. The plants were carefully removed from the medium, and Gelrite adhering to the roots was gently scraped off. The root and the shoot were dissected with a sharp scalpel. Observations on shoot and root lengths were taken. Roots were, stained with 0,1% Toulidine Blue (Sigma Chemical Co., St Louis, MO, USA) for 15 min and analyzed using WinRHIZO version 3,9, a scanner based image analysis system (Regent Instruments Inc., Quebec, Canada). The root samples were placed in plexiglass trays (200 X 300 mm) with a 3-4 mm deep layer of water to help untangle the roots and minimize root overlapping (Costa *et al.*, 2000). Data on total root length, total projected area, total surface area, average root diameter and number of tips were recorded. Leaf area was also measured with the same system, however, without staining. The roots and the shoots were then placed in paper bags and dried at 80 °C for 48h and then weighed by using an analytical balance.

Effects LCO in Combination with Verapamil and Chlorpromazine

The mediation of Ca^{2+} was evaluated in an experiment using combination of Ca^{2+} with verapamil (a plasma membrane Ca^{2+} channel blocker), and chlorpromazine (a CaM antagonist); and the optimal concentration of LCO (10^{-10} M) being used.

The above germination procedure was followed except that of verapamil (700 μ M) and chlorpromazine (70 μ M) were added to the medium before plating. The treatments evaluated were: LCO 10^{-10} M (1), Verapamil 700 μ M (2), LCO 10^{-10} M + Verapamil 700 μ M (3), LCO 10^{-10} M + Verapamil 700 μ M + 10 mM Ca^{2+} (4), Chlorpromazine 70 μ M (5), and LCO 10^{-10} M + Chlorpromazine 70 μ M (2). The experiment was arranged in a randomized complete block design with four replications of 10 seeds each.

Seed germinations were observed at 54 hour after treatment (about half of the seeds had germinated) and seedlings were harvested at 12 days after treatment. Seedling growth measured, including seedling dry weight (root, shoot and total),

root/shoot ratio, and seedling length (root, shoot and total).

Data were analyzed with ANOVA using the SAS Program (SAS version 6.12, SAS 1990, SAS Institute, NC, USA). Germination data were transformed into arcsin [(square root(x/10))] before analysis. The means of variables were separated by using LSD protected at 5%.

RESULTS AND DISCUSSION

The results of the experiment on the effect of LCO NodBj-V(C18:1,Mefuc) applied at 10^{-8} and 10^{-10} M on seed germination and seedling growth were presented in Table 1 and Figure 1, specifically on root growth on Table 2. LCO effects were significant on seed germination, seedling weight, total root length, root projected area, and root surface area; whereas on leaf area, and total number of root tips, though the effect were similar but not significant. However, root diameter was reduced by LCO treatment.

Although total seed germination was not affected (about 95% seed germinated), LCOs enhanced seed germination (Table 1, and Figure 1). Seed germination was significantly faster in LCO treated seeds than those in control seeds. T_{50} was reduced by 6 and 8 hours at 10^{-8} and 10^{-10} M from 58,7 h in the control seeds. There was an increase in dry weight of the seedlings after 10 days of incubation due to LCO treatment. Seedling dry weight was 5,12 mg in 10^{-10} M, being higher as compared with 4.07 mg in the control, but at higher concentration (10^{-8}), LCO effect was not significant. Similarly leaf area also increased as a result of LCO treatment.

The presence of LCO in the growth medium also improved root growth (Table 2). There was a significant increase in the total length of the roots. Both application of LCO at 10^{-8} and 10^{-10} M improve total root length, and again 10^{-10} M produced higher increase. The root lengths were 22.15 cm in 10^{-10} M LCO and 19.11cm in 10^{-8} M LCO versus 13.05 cm in the control. Similarly, there were increases in the total projected area and total surface area (Table 2). The patterns of increase in both the total projected area and total surface area were similar to those of increase in total root length. The number of root tips, as an

indicator for the extent of root branching, were doubled in 10^{-10} M LCO treatment, but it was not significantly higher than those in control ($p < 0.08$).

Addition of verapamil along with LCO reduced the germination and reduced the development of the plant as evidenced by reduced shoot length, root length and dry weight (Table 3). However addition of excess of calcium in the medium restored the effect of LCO. Similarly chlorpromazine also reduced the LCO enhancement of germination and seedling growth (Table 3).

The results of these experiments demonstrated that LCO accelerates the germination and early growth of cauliflower at

subnanomolar concentrations and that this effect is mediated by Ca^{2+} . Nod factors are potent inducer of cell division through the induction of cell cycle genes in plants. Purified nod factors, when targeted through a microballastic approach resulted in the induction of cell division at the site of contact (Schulaman *et al.*, 1997). Further, appropriate nod factors *per se* can induce *de novo* organogenesis of nodules in a variety of plants (Cohn *et al.*, 1998). Similarly, induction of cell division by nod factors in non-legume has been reported (Daychok *et al.*, 2000). Thus the observed increase in germination and early growth of cauliflower by LCO might be due to the LCO elicited increase in cell cycle activity.

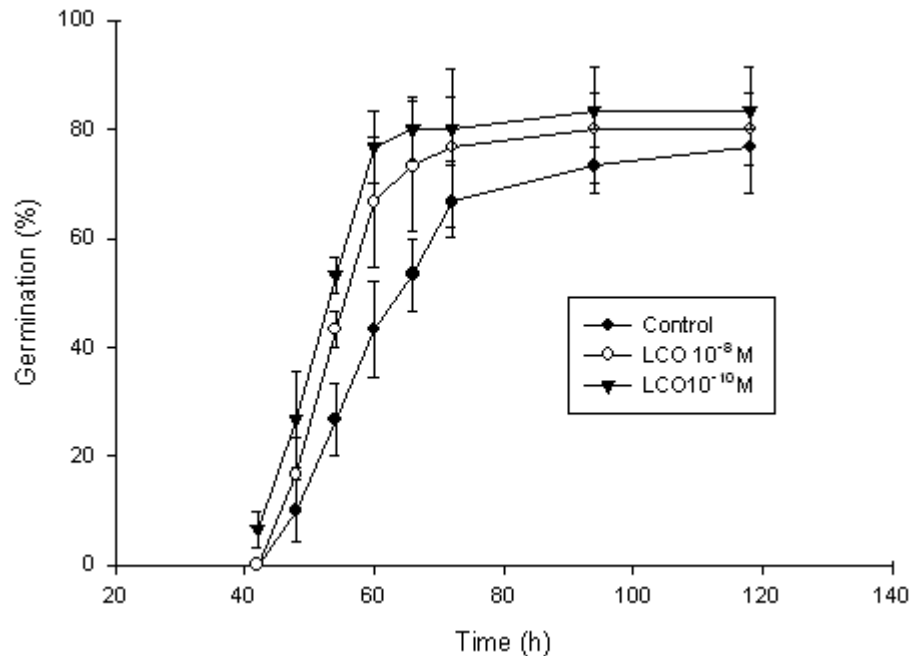


Figure 1. Effect of LCO (NodBj-V(C: 18, Mefuc)) on germination of cauliflower baers show standard errors

Table 2. Effect of Lipo-chitooligosaccharide (NodBj-V(C18:1,Mefuc)) on root growth of cauliflower

Treatment	Total length (cm)	Projected area (cm ²)	Surface area (cm ²)	Average diameter (mm)	Total number of tips
Control	13,05 b	0,74 b	2,32 b	1,76	28,0
LCO 10^{-8} M	19,11 a	1,12 a	3,51 a	1,50	49,5
LCO 10^{-10} M	22,15 a	1,24 a	3,89 a	1,14	56,7

Keterangan : Values within the same column followed by the same letter are not significantly different at LSD 0,05.

Table 3. Effect of Lipo-chitooligosaccharide (LCO), Verapamil, and Chlorpromazine on cauliflower seed germination and seedling growth

Treatment	Germination (%)	Length (mm)		Dry Weight (mg)		Total
	60 hours	Root	Seedling	Root	Shoot	
LCO 10 ⁻¹⁰ M	70	74,3 b	99,9 b	1,36 a	5,82 a	7,17 a
Verapamil	63	56,2 c	78,2 c	1,28 ab	4,99 abc	6,27 ab
LCO + Verapamil	57	54,3 c	77,3 c	1,25 abc	4,25 c	5,45 b
LCO + Verapamil + Ca	70	83,6 a	109,1 a	1,21 ab	5,07 abc	6,31 ab
Chlorpromazine	67	39,6 d	62,8 d	1,00 c	4,36 bc	5,36 b
LCO + Chlorpromazine	70	30,0 e	55,7 d	1,12 bc	5,14 ab	6,26 ab

Keterangan : LCO were applied at 10⁻¹⁰ M, Verapamil at 700 μ M, Ca²⁺ at 10 mM, and Chlorpromazine at 70 μ M. Seedlings were harvested at 12 days of planting. Values within the same column followed by the same letter are not significantly different LSD_{0,05}

Given the minute concentration of LCO required for the observed effects it is reasonable to assume the presence of specific receptors for LCO in the plant. Two classes of putative receptors have been purified from microsomal fractions of alfalfa root tissues: NFBS1 has low substrate affinity while NFBS2 had higher affinity (Gressant *et al.*, 1999). These receptors did not discriminate between sulfuryl substitution needed for *in vivo* activity. Recently, internalization of LCO in plant cells have been reported (Timmers *et al.*, 1998; Philip-Hollingsworth *et al.*, 1997) suggesting that the perception of LCO signal in plant might be more complex. Further, the present experiments demonstrate that the effect of LCO is mediated by Ca²⁺. Several workers have reported the role of Ca²⁺ in transduction of LCO signal (Felle *et al.*, 1998, 1999; Yokoyama *et al.*, 2000). Our results are in conformation with the published works although our results form the first report of the mode of action of LCO in a non-legume whole plant system.

Nod factor responsive genes have been characterized in a non-legume, rice (Kouchi *et al.*, 1999; Reddy *et al.*, 1998). Thus it seems that perception of nod factor might be conserved among wide variety of plants and the present results argue for the above contention. Further, the present result opens the possibility of using this novel class of signal molecule for improving plant growth in a wide range of plants.

CONCLUSION

Based on data obtained in the experiments, we conclude that germination of Cauliflower seeds are modified by application of LCO in

concentration manner, being optimum at 10⁻⁸ M and 10⁻¹⁰ M. LCOs increase germination speed (T₅₀), total root length, and total surface/projected area of the roots. Leaf area, hypocotyl length and seedling weights are not affected by LCO treatment. Pharmacological evidences showed that an addition of verapamil or chlorpromazine to the LCO's medium nullified the positive effects of LCO, suggesting the role of Ca²⁺ in the signal transduction of LCO activity. In addition, excessive amount of Ca²⁺ in the medium overcame the inhibitory effect of verapamil. We conclude that Ca²⁺ may play a role as a second messenger in signal transduction for growth induction of LCO.

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