MOLECULAR EVENTS IN *NICOTIANA TABACUM* AND *GLYCINE MAX* FOLLOWING LIPOCHITOOLIGOSACCHARIDE TREATMENT

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TABLE OF CONTENTS

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ABSTRACT

Lipochitooligosaccharides (LCOs) are molecules secreted by rhizobia during the establishment of rhizobia-legume N_2-fixing symbiosis. Some recent reports have shown that there are physiological effects on host and non-host rhizobia after LCO treatment. However, the cellular mechanisms underlying these observations are not known. Therefore, the study was aimed at assessing phenotypic changes by measuring photosynthesis, leaf area and dry weight on a non-host plant (tobacco). Our results did not show any significant physiological changes following LCO treatment. We also wanted to explore the molecular basis of changes in the plant cell by looking at gene and protein profiling following LCO treatment in a natural host plant (soybean) using real-time RT-PCR and SDS-PAGE. To do so, a reproducible stimulation method for soybean seed germination by LCO was successfully developed. The results obtained on soybean did not reveal significant differences in gene expression between water and LCO-treated seeds for the genes *cdc2*, *WASI*, *ICL1* and *14-3-3* studied. In addition, LCO treatment did not change the protein profile compared to the water control in a significant way.

RESUME

Les lipochitooligosaccharides (LCOs) sont des molécules sécrétées par des bactéries du genre rhizhobium lors de l'établissement d'une relation symbiotique avec des légumineuses. Il a été démontré que l'application de LCOs provenant de *B. japonicum* sur des plantes hôtes ou non hôtes entraînait divers changements physiologiques. Cependant, les mécanismes cellulaires sous-jacents à ces observations demeurent inconnus. Les effets phénotypiques liés au traitement LCOs ont été étudiés sur le tabac, une plante non hôte, en mesurant la photosynthèse, la surface foliaire et le poids sec. Ces expériences n'ont montré aucun changement physiologique suite au traitement. Les mécanismes moléculaires impliqués dans les phénomènes observés chez les plantes hôtes ont été explorés en étudiant le profil d'expression génique et protéique du soya suivant un traitement LCOs en utilisant le PCR en temps réel et le SDS-PAGE. Les résultats obtenus sur le soya n'ont révélé aucune variation significative dans l'expression génique des graines traitées aux LCOs pour les gènes *cdc2*, *WASI*, *ICL1* et *14-3-3*. De plus, le profil protéique du soya traité aux LCOs n'a pas été modifié de façon significative comparativement au contrôle. Une méthode a été développée afin de stimuler la germination du soya par l'application de LCOs.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

LCO	Lipochitooligosaccharide
°C	Degree Celsius
PCR	Polymerase Chain Reaction
Eq	Equation
CP	Crossing Point
Sec	Second
bp	Base pairs
Kb	Kilo base pair
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
mM	Millimolar
µM	Micromolar
ml	Milliliter
µl	Microliter
g	Earth's gravitationnal force
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
BSA	Bovine serum albumin
NaCl	Sodium chloride
$MgCl_2$	Magnesium chloride
Taq	*Thermophilus aquaticus*
EDTA	Ethylenediamine tetra acetic acid
TBE	Tris-boric acid EDTA

INTRODUCTION

1. General introduction

The establishment of an interaction between rhizobia and host plants requires the recognition of specific signal molecules by each partner. Initially, plants excrete flavonoids (Schlaman et al., 1998), leading to an induction of transcription of bacterial nodulation genes. Lipochitooligosaccharides (LCOs) are secreted by the bacteria in response to flavonoid induction (Lerouge et al., 1990). This molecular exchange finally leads to symbiosis.

Some recent reports evoke the possibility of using LCOs to improve yield in crop production. The major Nod factor of *B. japonicum* was found to promote early growth of soybean and corn in the greenhouse (Souleimanov et al., 2002). It has been shown that the irrigation of non-host plant seeds from diverse botanical families with synthetic LCOs leads to a stimulation of germination and an augmentation of biomass (Prithiviraj et al., 2003; Souleimanov et al., 2002). It has been demonstrated that Nod factor enhances photosynthesis under greenhouse and field conditions (Donald L. Smith, personal communication; Khan et al., in press). However, the cellular mechanisms underlying these observations are not known. Therefore, we wanted to explore the molecular basis of changes in the plant cell following application of LCO.

Research done previously provided indications that the physiological changes associated with LCO application might result from its influence at the transcriptional level. Nod factors induce the expression of genes early in nodule development. Many genes identified on the basis of their induced or enhanced expression during nodule development appear to encode cell wall proteins (Schultze and Kondorosi, 1998). Examples include genes coding for the proline-rich proteins Enod2, Enod5, Enod10, Enod11, Enod12, PRP4 (Hirsch and LaRue, 1997; Munoz et al., 1996; Mylona et al., 1995), extensins (Arsenijevic-Maksimovic et al., 1997), glycine-rich proteins (GRPs) (Küster et al., 1995) and a peroxidase (*ripl*) (Cook et al., 1995; Peng et al., 1996). The

earliest Nod factor-induced genes are *enod12* and *ripl* which are associated with the pre-infection stage and are expressed in nodule primordia and root hairs. The cdc2 gene, involved in cell division cycle, is induced in alfalfa cortical cells which divide after inoculation with *Rhizobium*, whereas cdc2 mRNA is not detectable in cortical cells of uninoculated roots (Yang et al., 1994). The same research (Yang et al., 1994) showed that histone H3, which is part of the core histone, is also induced in cortical cells inoculated with *Rhizobium.*

2. Hypotheses

Lipochitooligosaccharides increase the rate of photosynthesis per unit leaf area under greenhouse and field conditions (Donald Smith, personal communication) and increase biomass (Souleimanov et al., 2002). The hypothesis is that foliar application of LCO on host and non-host plants will modify gene expression by increasing transcription of cell division cycle genes and by increasing transcription of starch biosynthesis genes. The plant *N. tabacum cv. Xanthii,* which is a non-host, will be used for these experiments. It was previously demonstrated that Nod factors enhance seed germination of different crops such as soybean and maize (Prithiviraj et al., 2003). The hypothesis is that LCO application on a host and non-host seed will modify gene expression that correlates with a stimulation of germination by the treatment. The expression of genes involved in seed germination such as storage breakdown (Rylott et al., 2001) and signal transduction pathways that play a role in the germination process (Testerink et al., 1999) is expected to increase. Soybean seed [*Glycine max* (L.) Merr] cv. OAC Bayfield will be used to perform these analyses. To investigate these hypotheses, gene expression will be studied using real-time RT-PCR on plants or seeds that have or have not been treated with the LCO Nod Bj-V (C18:1, MeFuc) from *Bradyrhizobium japonicum*. The third hypothesis is that LCO application will induce changes in protein expression.

3. Objectives

The main objective of this M.Sc project is to relate the phenotypic changes resulting from the application of LCO to changes in plant or seed transcription or translation patterns. Specific objectives are to:

1. Detect the LCO phenotypic effects on tobacco by measuring photosynthesis, leaf area and dry weight.
2. Develop a method to have a reproducible stimulation of soybean seed germination by LCO.
3. Study gene expression for genes that might be involved in the response to LCO.
4. Study protein profiling by using SDS-PAGE.

LITERATURE REVIEW

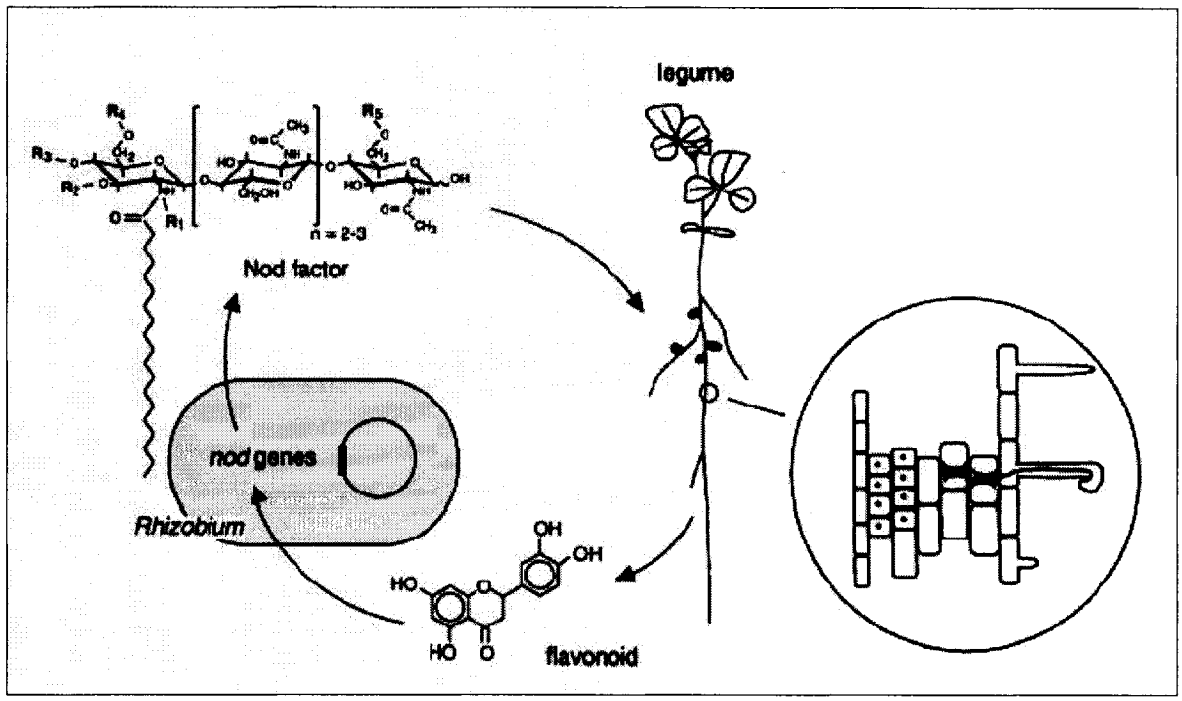
1. General description of lipochitooligosaccharides

1.1 Introduction

The interaction between bacteria belonging to the genera *Rhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium* (De Lajudie et al., 1998), collectively called rhizobia, with their host plants is conducted by specific recognition of signal molecules by each partner. In the first step, plants excrete compounds, usually flavonoids (Schlaman et al., 1998), that induce the transcription of bacterial nodulation genes called *nod* or *nol* genes. In the second step, bacteria produce and secrete Nod factors or lipochitooligosaccharides (LCOs) in response to flavonoid induction (Lerouge et al., 1990). The symbiosis leads to the formation of root, and sometimes stem nodules, on legumes (Fig. 1). Within these structures, the bacteria differentiated in bacteroids fix molecular nitrogen into ammonia or alanine (Waters et al., 1998) to be assimilated by the plant (Kaminski et al., 1998). During photosynthesis, the plant reduces carbon dioxide into sugars and translocates these to the root where the bacteria use them as a source of carbon and energy (Kahn et al., 1998).

1.2 Lipochitooligosaccharides structure and host specificity

All Nod factors synthesized by different rhizobia have the same general structure, usually three to five β-1,4-linked N-acetyl-D-glucosamine (GlcNAc) residues with a terminal non-reducing sugar residue having an N-acetyl group with an N-fatty-acyl (C16-18) chain. The *NodD* gene is found in all *Rhizobium*. Nod D protein is a transcriptional activator belonging to the LysR family and implicated in Nod factor production induced by specific flavonoids (Horvath et al., 1987; Schlaman et al., 1998; Spaink et al., 1987).



(From Schultze and Kondorosi., 1998)

Figure 1. Signal exchange in Rhizobium-plant symbiosis. Flavonoids induce transcription of rhizobial nod genes leading to the production of species-specific Nod factors or lipochitooligosaccharides (LCOs). Different substituents, such as methyl, carbamoyl, acetyl or sulphate can be found on the LCO backbone. LCOs produce different phenotypic responses on host plants, such as root hair deformation and cortical cell division. Eventually, the bacteria colonize the root surface of the plant and penetrate the cell where they differentiate into bacteroids in the new plant organ, the nodule. Under conditions of nitrogen limitation, the bacteroids fix molecular nitrogen which is used by the symbiotic plant (Schultze and Kondorosi, 1998).

The interaction of NodD with a specific host plant signal constitutes the first level of host-specific recognition (Dénarié et al., 1992; Spaink, 1995; van Rhijn and Vanderleyden, 1995). The *nodABC* gene products play a pivotal role in the synthesis of the LCO-backbone structure and are common to all rhizobial species. Mutations in one of these genes alter the host nodulation process; *nodABC* genes are required for bacterial cell division (Dudley et al., 1987) and deformation of root hairs (Bender et al., 1987; Djordjevic et al., 1985; Kondorosi et al., 1984; Rossen et al., 1984). Each rhizobial strain produces a characteristic Nod factor profile that seems adapted to the host plant. When isolated from the same host plant, the rhizobia belonging to diverse taxonomic groups produce LCOs of similar structure (Lorquin et al., 1997). Different *nod* genes are involved in modifying the basic LCO structure for different rhizobia to permit host specificity (Schultze et al., 1992; Spaink et al., 1991). These modifications include the attachment of sulphate, acetate, carbamoyl groups, addition of other sugars such as arabinose, mannose or fucose (and substituted derivatives of fucose), changes to the acyl chain, and variation of the chitin oligomer length (Downie and Walker, 1999; Dénarié et al., 1996). The next step in the symbiosis is the perception of Nod factors by the host plant.

1.3 Nod factor binding sites

Nod factors are amphiphilic molecules with hydrophobic acyl and hydrophilic oligosaccharidic moieties. Studies using fluorescently labeled LCOs suggest that Nod factors exist as monomers in aqueous solutions at concentrations up to at least 10 nM, rather than forming micelles through their hydrophobic acyl chains (Goedhart et al., 1999). This group demonstrated that Nod factors integrate spontaneously in biomembranes and transfer rapidly between membranes and to root hairs, probably by a mechanism involving desorption of monomers. However, transbilayer flip-flop does not occur and therefore the LCOs cannot spontaneously enter the plant cell without a specific recognition and transport mechanism. Plant responses can be induced by Nod factors at concentrations as low as 10^{-12}-10^{-13} M. This suggests the probable existence of high-

affinity receptors. There is indirect evidence for the uptake of LCOs into plant cells (Philip-Hollingsworth et al., 1997; Timmers et al., 1998) raising the possibility that Nod factor recognition could occur within cells as well as at the membrane surface. Following the analysis of infection by rhizobial mutants unable to make a Nod factor backbone fully substituted, it is suggested that there may be different types of Nod receptors, one controlling early responses and another controlling invasion events (Ardourel et al., 1994; Geurts et al., 1997).

Nod Factor Binding Site 1 (NFBS1) was first isolated from *Medicago truncatula* root extracts (Bono et al., 1995). It has an affinity of 86nM for the lipochitooligosaccharidic structure, but does not discriminate between the different substitutions on the oligosaccharidic backbone of the *R. meliloti* Nod factors which are important determinants of the host specificity of the *Rhizobium-Medicago* interaction (Ardourel et al., 1994; Roche et al., 1991). NFBS2, located at least partially in the plasma membrane of *M. varia* cell cultures, has both a higher affinity (4nM) and a greater selectivity for Nod factors. It recognizes the O-acetyl group and the hydrophobicity of the acyl chain but does not discriminate between sulphated and non-sulphated factors (Gressent et al., 1999; Niebel et al., 1997; Niebel et al., 1999). Recently, a lectin from roots of the legume *Dolichos biflorus* that appears to bind Nod factors was identified (Etzler et al., 1999). This lectin is an enzyme that catalyzes the hydrolysis of phosphoanhydride bonds of nucleoside di- and triphosphates. This *l*ectin-*n*ucleotide *p*hosphohydrolase (LNP) is present at the surface of root hairs, and treatment of roots with antiserum to LNP inhibits their ability to undergo root hair deformation and to form nodules upon exposure to rhizobia. When the soybean lectin gene *Le1* is introduced into *Lotus corniculatus*, which is normally nodulated by *R. loti*, a change in nodulation specificity occurs (van Rhijn et al., 1998). Nodule-like outgrowths are found on the transgenic plant roots in response to *B. japonicum* that usually nodulates soybean. Another class of candidate receptor genes are those whose activity depends on Nod factor structure, such as *SYM2* of pea (Albrecht et al., 1999). This interaction is evocative of host-pathogen gene-for-gene interactions, as the bacterium requires a *nodX* gene for nodulating plants with the *SYM2a* allele. Because *nodX* confers *O*-acetylation of the

reducing sugar of Nod factors, there is clearly a relationship with Nod factor perception (Albrecht et al., 1999). From detailed cytological studies of the block in nodulation, it has been argued that *SYM2* may encode a receptor for Nod factors that negatively regulates infection-thread growth (Geurts et al., 1997). Following recognition, Nod factors induce host responses.

2. Effects of Nod factors on plants

2.1 Nod factor effects on host plant physiological processes

Nod factors induce phenotypic responses from host plants like root hair deformation (Spaink, 1996), ontogeny of complete nodule structures (Dénarié and Cullimore, 1993; Fisher and Long, 1992), development of preinfection threads and cortical cell divisions (Sanjuan et al., 1992) (Table 1). This indicates that LCOs are mitogenic and morphogenic agents and present similar effects as cytokinins or inhibitors of auxin transport (Relic et al., 1993). In addition, LCOs are able to activate defense-related enzymes (Inui et al., 1997) and induce host nodulin genes essential for infection thread formation (Horvath et al., 1993; Minami et al., 1996). A rearrangement of actin microfilaments in bean (*Phaseolus vulgaris*) root hairs are observed in response to Nod factors from *R. etli* (Cardenas et al., 1998). Root hair deformation is caused by the reinitiation of tip growth in root hair cells that are about to cease elongation (De Ruijter et al., 1998; Heidstra et al., 1994). One of the very early events in root hairs induced by Nod factors is the rapid and transient plasma membrane depolarization (Ehrhardt et al., 1995; Felle et al., 1995; Kurkdjian, 1995). Moreover, a rapid alkalinisation of the root hair cytoplasm and of the root hair surface in a dose dependent manner is observed (Felle et al., 1996). Nod factors induce transient elevation of the cytosolic Ca^{2+} concentration in protoplasts generated from soybean roots or suspension-cultured microcallus cells (Yokoyama et al., 2000). Also, LCOs induce increases in intracellular calcium that are localized mostly around the nucleus (Ehrhardt et al., 1996). These increases occur about once per minute and the rapid rise and decrease in Ca^{2+} defines these as Ca^{2+} spikes.

Table 1. Responses of legume roots to Nod factors.

Tissue	Response	Rapidity of response	Concentration of Nod factors applied	Tested plants
Epidermis	Ion fluxes	Seconds	nM	*Medicago*
	Plasma membrane depolarization	Seconds	nM	*Medicago*
	Increase in intracellular pH	Seconds	nM	*Medicago*
	Accumulation of Ca^{2+} in root hair tip	Seconds	nM	*Medicago, Vigna*
	Ca^{2+} spiking	10 mins	nM	*Medicago, Pisum*
	Gene expression (e.g. *ENOD12, RIP1*)	Mins–hours	fM–pM	*Medicago*
	Root hair deformation	Mins–hours	nM–μM	Many
	Cytoskeleton modification	Mins–hours	fM–pM	*Phaseolus, Vicia*
Cortex	Gene expression (e.g. *ENOD20*)	Hours–days	pM	*Medicago*
	Formation of pre-infection threads	Days	nM–μM	*Vicia*
	Cell division leading to nodule primordium formation	Days	nM–μM	Many
Vasculature	Inhibition of polar auxin transport	Mins	nM–μM	*Trifolium*
	Gene expression (e.g. *ENOD40*)	24 hours–days	nM–μM	*Glycine, Vicia, Medicago*

(From Cullimore et al., 2001)

The spiking initiates ~10 minutes after Nod factor addition on alfalfa and continues for at least 3hrs (Ehrhardt et al., 1996). Some evidence suggests a role for Ca^{2+} as a secondary messenger in signal transduction of Nod factors (Felle et al., 1999). Schlaman *et al.* (1997) demonstrated that *O*-acetylated chitin oligosaccharides can induce root cortical cell divisions of *V. sativa*. They proposed that chitin oligosaccharides act indirectly on their target cells *via* a secondary signal transduction pathway, which is activated as soon as the oligosaccharide enters the root tissue. Some evidence showed the involvement of G protein in Nod factor signal transduction (Kelly and Irving, 2003; Pingret et al., 1998). Inner cortical cell division may result from a local alteration in the auxin/cytokinin hormone balance, for instance by influencing hormone transport across the endodermis, giving rise to local cell divisions (Schlaman et al., 1997). This hypothesis is supported by the investigations of different groups (Bauer et al., 1996; Hirsch et al., 1989; Mathesius et al., 1998; Rohrig et al., 1995). Schlaman et al. (1997) favored a model in which the oligosaccharide moiety of the rhizobial LCO induces cortical cell division and the fatty acyl moiety plays a role in transport of the LCO into the plant tissue. During the establishment of the *R. meliloti*-alfalfa symbiosis, the *Rhizobium*-synthesized Nod factors are possibly involved in the inhibition of salicylic acid-mediated defense in legumes (Martinez-Abarca et al., 1998).

2.2 Nod factor responses to host and non-host rhizobia

Some recent reports show that other phenotypic changes, in addition to these described in section 2.1, are observed in the host. It was demonstrated that LCOs can also have a direct impact on non-host plants without the implication of bacteria and nodulation process. It was found that cell division and embryo development can be restored in a temperature-sensitive carrot mutant by Nod factors (De Jong et al., 1993). A rapid and transient alkalinization of tobacco (Baier et al., 1999) and tomato cells (Staehelin et al., 1994) is provoked by Nod factors when added to suspension cultures. Rhizobial nod gene products are shown to modulate tobacco development (Schmidt et al., 1993). Synthetic LCOs are plant growth regulators and promote cell division at 10^{-15} M concentrations in tobacco protoplasts grown in the absence of auxin and cytokinin

(Rohrig et al., 1995). Synthetic LCOs can therefore alleviate the requirement of the phytohormones auxin and cytokinin to sustain growth of tobacco protoplasts. Cell division and somatic embryogenesis is promoted by LCOs in a conifer, Norway spruce (*Picea abies*) in the absence of auxin and cytokinin, suggesting that rhizobial Nod factors substitute for conditioning factors in embryogenic cultures (Dyachok et al., 2000). The major Nod factor of *B. japonicum* was found to promote early growth of soybean and corn in the greenhouse (Souleimanov et al., 2002). It was shown that the irrigation of host and non-host plant seeds from diverse botanical families with LCOs leads to an augmentation of biomass (Prithiviraj et al., 2003; Souleimanov et al., 2002). The studies also showed that soybean shoots and roots treated with LCOs were longer compared to the control. Moreover, Nod factor treatments stimulated branching of soybean roots and the plants produced more pods (Souleimanov et al., 2002). Recently, it was demonstrated that LCOs can stimulate germination of a variety of economically important plants from diverse botanical families such as *Zea mays*, *Oryza sativa* (Poaceae), *Beta vulgaris* (Chenopodaceae), *Glycine max*, *Phaseolus vulgaris* (Fabaceae) and *Gossypium hirsutum* (Malvaceae) (Prithiviraj et al., 2003). The group also found an increase in α-amylase activity in corn seeds treated with LCOs. They hypothesized that the rapid germination and seedling growth rate may be the result of an enhancement of the cell cycle rate by LCO application. It was demonstrated that Nod factor enhances photosynthesis under greenhouse and field conditions (D.L. Smith, personal communication). The cellular mechanisms underlying these observations are not known.

3. Plant molecular events induced by Nod factors

3.1 Plant genes induced during nodulation process

Nod factors induce the expression of early genes in nodule development. Many genes identified on the basis of their induced or enhanced expression during nodule development appear to encode cell wall proteins. This underlines the important role that alterations in cell wall biochemistry must play during infection and nodule development

(Schultze and Kondorosi, 1998). Examples include genes coding for the proline-rich proteins Enod2, Enod5, Enod10, Enod11, Enod12, PRP4 (Hirsch and LaRue, 1997; Munoz et al., 1996; Mylona et al., 1995), extensins (Arsenijevic-Maksimovic et al., 1997), glycine-rich proteins (GRPs) (Küster et al., 1995) and a peroxidase (*ripl*) (Cook et al., 1995; Peng et al., 1996). The earliest Nod factor-induced genes are *enod12* and *ripl*, which are associated with the preinfection stage and are expressed in nodule primordia and root hairs. Futhermore, Nod factors can elicit the expression of chalcone synthase (*CHS*) (Lawson et al., 1994), chalcone reductase, isoflavone reductase as well as genes encoding a pathogenesis-related protein in *Medicago* suspension cultures (Savouré et al., 1997). Gene transcription of *M. truncatula ENOD12* was shown to occur as early as 3 to 6hrs following inoculation with *R. meliloti* in a zone of differentiating root epidermal cells which lies close to the growing root tip (Pichon et al., 1992).

Later on, it was demonstrated that purified LCOs induced the expression of the pea early nodulin genes, *PsENOD5* and *PsENOD12* (Horvath et al., 1993). Cell specific transcription of the *ENOD12* gene in transgenic alfalfa was elicited by the *R. meliloti* Nod factor (Journet et al., 1994). It was reported recently that a white clover nodulin gene, *dd23b*, is expressed in roots after treatment with Nod factors from *R. leguminosarum* biovar *trifolii* (Crockard et al., 2002). *In situ* hybridization analysis showed that the early nodulin gene *ENOD40* mRNA is abundant in root pericycle cells before the appearance of infection threads in soybean (Kouchi and Hata, 1993; Yang et al., 1993). The expression of *ENOD40* was also detected in the pericycle cells surrounding vascular strands in mature nodules by Kouchi et al. (1993) and Yang et al. (1993). *ENOD40* mRNA was abundant at 40hrs after inoculation of soybean roots with LCOs (Minami et al., 1996). *ENOD40* induced the de-differentiation and division of root cortical cells in legumes (Charon et al., 1997). Interestingly, *ENOD40* gene homologues were found previously in tobacco (van de Sande et al., 1996) and in rice (Kouchi et al., 1999).

The gene *VsLbI* encodes a leghemoglobin and is induced in vetch roots within 1 hr after Nod factor application (Heidstra et al., 1997). Recently, three genes were estimated to be up-regulated by using differential display at 6hrs after initiation of treatment. The

soybean suspension-cultured cells were treated with Nod factor obtained from *B. japonicum* (Hakoyama et al., 2002). The group also found that four genes were down-regulated. The transcriptional level of the seven genes studied was clearly down-regulated within 2hrs after initiation of treatment. *R. meliloti* Nod factor can modify gene expression of *Medicago* microcallus suspensions. LCOs stimulated cell cycle progression as indicated by the increase in kinase activity of the $p34^{cdc2}$-related complexes and enhancement of the level of expression of several cell cycle marker genes: the histone H3-1, the cdc2Ms and the cyclin cycMs2 (Savouré et al., 1994). Savouré et al. (1994) also found that a high concentration (10^{-6} M) of Nod factor could induce the expression of the isoflavone reductase gene (*IFR*), a gene that is part of the isoflavonoid biosynthesis pathway. Therefore, cyclin and histone genes are good molecular markers to study the induction of mitosis in cortical cells by *Rhizobium* and nodulation factors (Yang et al., 1994).

3.2 Protein stimulated by Nod factors

In response to LCO treatment, tobacco protoplasts were found to release a second growth factor, a peptide, that had the ability to stimulate cell division in the same way as extracellular LCOs stimulus (John et al., 1997). Evidence indicated that the LCO-induced mitogen released by tobacco cells may be related to the region 2-derived peptide of the tobacco homologue early nodulin gene *ENOD40*. There are few reported studies on the direct effect of LCOs on plant proteins. LCOs usually act on transcriptional level and the difference is seen in protein profiling.

3.3 Impact of LCOs on gene expression on non-host

LCOs increased the levels of *AXI1* transcripts in tobacco protoplasts grown in absence of auxin. The *AXI1* gene is auxin and cytokinin responsive and its deregulated expression uncouples protoplasts from the normal effects of auxin on cell division (Hayashi et al., 1992). LCOs and auxin are transduced within the tobacco cell via

separate pathways that converge at or before *AXI1* transcription. To reach full growth-promoting activity, both LCOs and auxin also require the presence of the effector cytokinin (Rohrig et al., 1996). Cytokinin is suggested to be a common effector of LCOs and auxin signaling. Reddy et al. (1998) introduced the promoter of the infection-related gene *ENOD12* from *M. truncatula* fused to the β-glucuronidase reporter gene into rice. *MtENOD12*-GUS gene expression was induced in cortical parenchyma, endodermis and pericycle of the transgenic rice plant when the roots were treated with LCOs under nitrogen starvation (Reddy et al., 1998). The results suggest that at least a portion of the signal transduction machinery essential during the nodulation is present in rice.

MATERIAL AND METHODS

1. *Variation of gene expression of* Nicotiana tabacum *cv.* Xanthii *after lipochitooligosaccharide treatment*

1.1 Plant growth conditions

N. tabacum cv. *Xanthii* plants were grown from seed in a large tray in Promix (Premier Tech, Rivière-du-Loup, Québec, Canada) in a greenhouse. Seedlings were transferred to 2.5 cm pots in Promix. Plants were watered as necessary and were fertilized every week with 50 ml of 20-20-20 K/P/N at 3 g/L.

1.2 Plant treatment

Plants were sprayed at the 3-5 leaf stage. Both sides of the leaf were sprayed until saturation. Control plants were sprayed with distilled water and 0.02% of Tween 20. LCO-treated plants were sprayed with a solution of 0.02% of Tween 20 and 10^{-8} M of LCO Nod Bj-V (C18:1) MeFuc from *B. japonicum* strain 542C, using peak B. The 10^{-8} M concentration was determined according to the optimal concentration used in previous work (Prithiviraj et al., 2003). LCO was provided by Dr. Donald L. Smith (McGill University, Québec, Canada). The purification of LCO was performed by HPLC as described by (Prithiviraj et al., 2000).

1.3 Photosynthetic measurement

The LICOR LI-6200 photosynthesis system (LI-COR, Inc., Lincoln, Nebraska, USA) was used to measure photosynthesis of each plant by using the youngest leaf that

was fitted in the chamber (6 cm^2). The photosynthetically active radiation inside the chamber was 400 or 800 μmol m^{-2} sec^{-1} depending on the light intensity in the greenhouse. The chamber CO_2 concentration was set at 400 ppm. Measurements were taken when the coefficient of variation of CO_2 was below 0.4%. The experiment was conducted every day at the same time. The first measurement at day « 0 » was done before the plants were sprayed with the LCO.

1.4 Total leaf area and dry weight measurements

Tobacco leaf area and dry weight were measured 15 days after the plants were treated as described in section 1.2. These measurements were made 15 days after treatment application according to previous work (Prithiviraj et al., 2003). Total leaf measurement was done on each sample using an Area Measurement System (Delta-T Devices Ltd., Cambridge, England). The leaves and stem of the plants were then dried for 72hrs at 90°C ± 1 in paper bags and weighted.

2. *Variation of gene expression of* Glycine max *(L.) Merr] cv. OAC Bayfield after LCO treatment*

2.1 Seed germination

A seed germination test was used to verify if the LCO was biologically active. The test was performed in a 9 cm diameter sterile plastic Petri dish (Fisher Scientific Canada, Ontario) with one sterile filter paper disk Whatman 1 (Whatman Inc., Ann Arbor, Michigan, USA) at the bottom. For the germination of soybean [*G. max* (L.) Merr] cv. OAC Bayfield seeds, 7 ml of water or LCO solution were used per plate. Seeds were sterilized with 1.2% sodium hypochlorite for two minutes, washed with distilled water five times and dried on a paper. Seeds were then selected, ensuring that the

tegument was intact and the size of the seeds was uniform. Each Petri dish contained 10 seeds and each treatment had 10 replicates. The plates were incubated at 24°C ± 1 in the dark for 21-24hrs. The seeds were considered germinated if the radicle emerged from the tegument. One-way analysis of variance (ANOVA) was used to assess statistical differences ($P<0.05$) in the germination tests and the least difference (LSD) method was used to determine significant differences among means. For gene expression studies, sample preparation was the same as described above. Soybean embryos, scutellums, radicles and small parts of the endosperms were collected from seeds from one Petri dish, pooled in a plastic bag and frozen at -70°C. The experiment contained ten replicates with ten seeds for each treatment. Seeds from the ten replicates were collected at different time points after the addition of water or 10^{-8} M LCO.

2.2 Extraction of seed total RNA and reverse transcription into cDNA

To proceed with RNA extraction of seed samples and the analysis of gene expression, the percentage of germination with LCO treatment had to be significantly higher than the control, indicating a stimulation of germination by the treatment. After the statistical analysis, RNA extraction of three replicates for each time point for each treatment was performed. Each extraction was performed using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's recommendations. 50 mg of soybean embryos, scutellums, radicles and small parts of endosperms from seeds not-treated and treated with LCO were ground in liquid nitrogen in a chilled mortar. Clean mortars were prepared by wrapping in aluminium foil and baking for at least 4hrs at 400°C. Two washes with solution RW1 (supplied in the Qiagen kit) were done before the DNase1 treatment and one extra wash after the treatment. DNase1 (Qiagen) digestion with 27,3 U in RDD buffer was carried out for 25 minutes on each sample. After RNA extraction, the optical density at 280 nm and 260 nm was measured for each sample with a spectrophotometer (Ultrospec 4300 Pro UV/Visible spectrophotometer, Biochrom Ltd.) to determine the purity of the RNA. Each sample had an $OD_{260/280}$ ratio ranging between 1.8-2.1. The concentration of each sample was determined by using the RiboGreen RNA

quantification kit (Molecular Probes Inc., Eugene, Oregon, USA). RNA samples were diluted 1/100 in 1X TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) supplied with the kit. Ribosomal RNA standards supplied with the kit were diluted similarly. Fluorescence at 530 nm was measured in a multiplate reader Synergy HT (BIO-TEK Instruments, Inc., Winooski, Vermont, USA). A standard curve was made by simple linear regression using the values obtained with the ribosomal RNA dilutions and the concentrations of the RNA samples were determined by interpolation. 1μg of total RNA was used to make cDNA using the Omniscript Reverse Transcriptase kit (Qiagen). The RT step was primed using oligo-dT primers (Amersham Biosciences, PlusOne, Baie-d'Urfé, Québec, Canada) because these primers maximize the number of mRNA molecules that can be analyzed from a small sample of RNA (Bustin, 2000). To ensure that the sample was not contaminated with DNA during the RNA extraction, each sample had a control that consisted of the same components without the reverse transcriptase.

2.3 Testing the quality of each sample by PCR

Each cDNA sample was first analyzed by the polymerase chain reaction (PCR) to verify the quality of the sample and check whether one sharp band was obtained with all primer sets with the conditions described above (Table 2). The Primer 3 software (www-genome.wi.mit.edu/cgi-bin/primer/ primer3_www.cgi) was used to design the primers based on sequences from the National Center for Biotechnology Information website (NCBI) corresponding to the gene needed. PCR reactions were performed in a total of 25 μl containing 1X of PCR buffer 10X (Gibco BRL), 200 μM of dNTPs (Invitrogen Life Technologies, Burlington, Ontario, Canada), 1.5 mM of $MgCl_2$ (Invitrogen Life Technologies), 1.25 U of *Taq* DNA polymerase (Invitrogen Life Technologies), 200 μM of each forward and reverse oligosaccharides and 50 ng of cDNA. The GeneAmp PCR System 9700 (Perkin Elmer/Applied Biosystems, Foster City, California, USA) was used to amplify templates and conditions were set as follows: 5 minutes at 94°C, then 30 cycles of denaturing for 30 seconds at 94°C, annealing for 45 seconds at 55°C, and primer extension for 1 minute at 72°C. These conditions were used for *cdc2*, *WASI*, *ICL1*,

Table 2. Genes and primer sets designed on soybean sequences used with PCR and real-time RT-PCR.

Genes	Product	Sens	Sequences	Accession number	Annealing temperature	Product size
cdc2	cell division cycle protein 2	Forward	5' TTCCCAGATCGTCTTCTTCC 3'	BU761018	55°C	174 bp
		Reverse	5' GAGGCGAATCTTCTTCAACG 3'			
gene 14-3-3	14-3-3 protein	Forward	5' CATTAGGCTGGGACTTGCTC 3'	AF532628	55°C	206 bp
		Reverse	5' CCGTGATGTCTGATGTCCAC 3'			
ICL1	isocitrate lyase	Forward	5' GTGGCAGTTCATCACTGTGG 3'	CA800911	55°C	203 bp
		Reverse	5' CTCCCTGGACAGTCTTGAGG 3'			
WASI	alpha amylase inhibitor	Forward	5' TCGTGTCCTTTGTACGTTGG 3'	CA936838	55°C	186 bp
		Reverse	5' ACCCACTCTCCATTCTGTGG 3'			
RPL13	60S ribosomal protein L13	Forward	5' AAAGACCAGAAGACGGTTGG 3'	CA820658	55°C	211 bp
		Reverse	5' GCGGTGATCAACAGAAATCC 3'			
gtub	gamma-tubulin	Forward	5' ACATGAAAGCGGTGATAGGG 3'	BE023133	57°C	181 bp
		Reverse	5' GTTTGGCTTCATGGTCTTCC 3'			

14-3-3 and *RPL13* primer sets. The 30 PCR cycles were followed by 5 minutes at 72°C and kept at 4°C. The ramping rate was 1°C per second for all steps. For the *gtub* primers, the PCR conditions were similar except that the annealing temperature was 57°C instead of 55°C. Each sample was run on agarose gel and stained with ethidium bromide to show the amplification products and test for the presence of DNA contamination.

2.4 Agarose gel electrophoresis of PCR and real-time RT-PCR products

Agarose gels of 1.5% w/v were prepared by melting the appropriate amount of agarose (Invitrogen Life Technologies) in 0.75X Tris-Boric EDTA (TBE) buffer [89 mM Tris-base, 89 mM boric acid, 2 mM EDTA (pH 8.0)]. Agarose gels dissolved in 0.75X TBE buffer were poured into a gel bed. PCR or real-time RT-PCR products were mixed with 6X loading buffer [60% glycerol, 60 mM EDTA, 0.2% xylene cyanol] and loaded into the agarose gel wells. Electrophoresis was performed in 0.75X TBE buffer using a 20 cm long gel at 3.2 V/cm and run for at least one hour. Electrophoresis products were stained with ethidium bromide (sensitivity 30 pg/band) at a concentration of 0.5 μg/ml in 0.75X TBE buffer for 15 minutes and visualized with a Molecular Imager FX (Bio-Rad Laboratories Inc., Mississauga, Ontario, Canada) at a resolution of 100 μm.

2.5 cDNA quantification on real-time RT-PCR

Reactions were performed using the LightCycler instrument (Roche Molecular Biochemical, Laval, Québec, Canada). The Quantitect SYBR Green PCR reagents (Qiagen) were used following the manufacturer's instructions. PCR was performed in a total volume of 20 μl in a glass capillary (Roche Molecular Biochemicals). The 2X Quantitect SYBR Green PCR Master Mix, supplied by Qiagen, contained HotStarTaq DNA polymerase, Quantitect SYBRGreen PCR buffer, dNTP mix, SYBR Green 1, ROX (passive reference dye) and 5 mM $MgCl_2$. A final concentration of 250 μM of each

forward and reverse primer was used along with 2 µl of cDNA diluted 1/10. The appropriate standard was used for each primer set with a range of concentration between 1 ng/µl and 0,0001 pg/µl constructed by a 10X dilution factor. All standards were prepared using the LightCycler and purified with QIAquick PCR purification kit (Qiagen). Reaction conditions varied for each primer set. A typical set of conditions consisted of the following steps: pre-incubation at 95°C for 15 minutes, amplification for 45 cycles (94°C for 15 sec, 55°C for 25 sec, 72°C for 20 sec and acquisition of data at 78°C for 1 sec), melting curve analysis: 95°C for 5 sec, 65°C for 15 sec and a stepwise acquisition of fluorescence data to 96°C at 0.1 sec intervals. The ramping rate was 10°C/sec for all steps. Reactions were rejected if amplification was detected in the water control or if less than four standard concentrations out of seven amplified properly.

2.6 Gene expression analyses

Expression of each gene was compared using three different methods. The first method was the comparison of gene expression using the crossing point (CP) of the control and the LCO 10^{-8} M treatment curves without any mathematical transformation of the data. This method of analysis did not include the correction for magnitude of gene expression using an internal reference. One-way analysis of variance (ANOVA) was used to test for statistical differences ($P<0.05$) and the least significant difference (LSD) method was used to determine significant differences among means. Statistical analysis was performed on the Statistical Analysis System (SAS Inc., NC, USA, 1989). The second method described by Pfaffl was a mathematical model using a relative expression ratio (Pfaffl, 2001). This method used an internal reference (Eq. [1]):

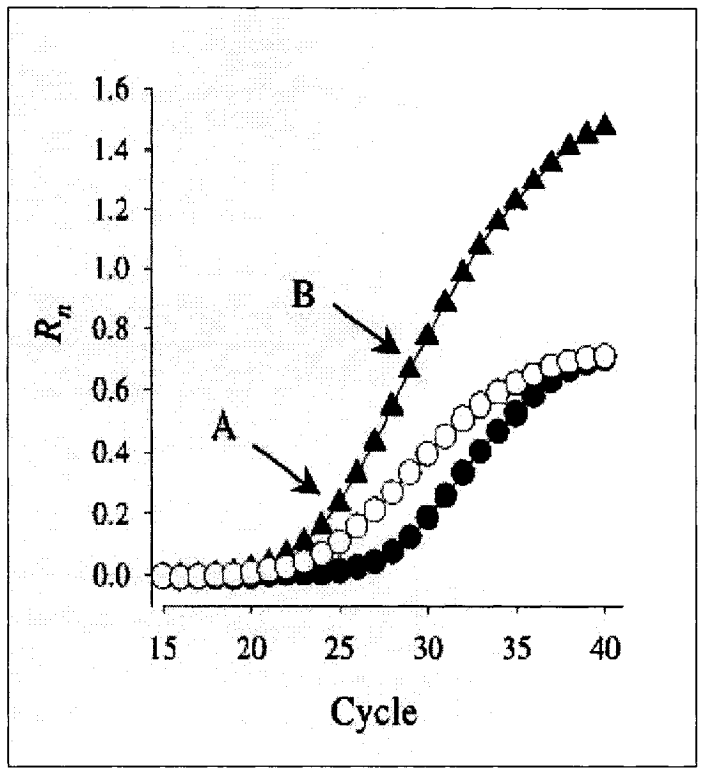
$$E = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}} \qquad [1]$$

Using the Pfaffl method, the ratio of a target gene was expressed relative to a control gene. E_{target} was the real-time PCR efficiency of the target gene transcript calculated according to the equation $E = 10^{[-1/slope]}$. The cDNA reference samples (with known concentrations) were plotted against CP cycles and the linear regression slope calculated. E_{ref} was the real-time PCR efficiency of a reference gene transcript. The slope was also calculated from a graph of CP cycles versus cDNA standard concentrations. Standards were analyzed in the same PCR reaction as the reference gene. The ΔCP was the difference of CP between control and sample gene transcripts. The use of standards was dependent on the assumption that the amplification efficiency of standard and cDNA samples was identical (Peirson et al., 2003). However, the standards were constructed following the same protocol as the cDNA samples.

The third method described by Liu et al. (2002) also used relative gene expression. Compared to the Pfaffl method that calculated the efficiency by using the slope from the standard curve (CP cycles versus cDNA standard concentration input), this method calculated the amplification efficiency from the kinetic curves of each sample individually (Liu and Saint, 2002). An example of calculation of amplification efficiency from the kinetic PCR curve from Liu et al. (2002) is presented in Figure 2. The PCR efficiency of each sample was calculated using the equation [2]:

$$E = -\left(\frac{R_{n,A}}{R_{n,B}}\right)^{-(C_{T,A}-C_{T,B})} - 1 \qquad [2]$$

where $R_{n,A}$ and $R_{n,B}$ are fluorescence at arbitrary thresholds A and B on the same sample curve and $C_{T,A}$ and $C_{T,B}$ are the threshold cycles at these arbitrary thresholds (Figure 2). In this case, CP values were used instead of C_T, which is the same as CP according to the definition. With this method, standards were not required to calculate the amplification



(From Liu et al., 2002)

Figure 2. An example of calculation of amplification efficiency from the kinetic PCR curve. A and B represent two arbitrary thresholds selected along the same sample exponential phase. $R_{n,A}$ and $R_{n,B}$ are the fluorescence measured for the two arbitrary thresholds A and B and $C_{T,A}$ and $C_{T,B}$ are the threshold cycles at these arbitrary thresholds. The PCR efficiency of each sample is calculated using Eq. [2].

efficiency. Sample transcript levels were then normalized using an internal control transcript level with equation [3] (Liu and Saint, 2002):

$$R_{O,T} \Big/ R_{O,R} = \frac{(1+E_R)^{C_{T,R}}}{(1+E_T)^{C_{T,T}}} \qquad [3]$$

where E is the sample amplification efficiency, C_T is the cycle at which PCR products begin to increase exponentially and added subscripts R and T represent reference and target genes, respectively. As shown in Eqs. [2] and [3], the difference in gene expression between the control and the LCO-treated seeds is not considered in these formulas. Therefore, the ratio of LCO treated and control samples was made in order to assess changes in gene expression.

Statistical analysis was performed on the ratio obtained using the Liu and Pfaffl transformation methods in order to assess the statistical difference between water and LCO treatments. The two-tailed t-test statistical analysis was used for μ (Eq. [4]):

H_0: $\mu = 1$ versus H_1: $\mu \neq 1$

$$t_{obs} = \frac{\left(\overline{X} - 1\right)}{\left(\frac{S}{\sqrt{n}}\right)} \qquad [4]$$

The H_0 hypothesis was rejected if the $|t_{obs}| > |t_{\alpha/2}|$ at a P-value of 0.05.

3. SDS-PAGE of soybean seed proteins

3.1 Protein extraction from soybean seeds

The soybean seeds treated or not treated with 10^{-8} M of LCO were germinated according to section 2.1 of material and methods. Samples were collected over time and were kept at -70°C until protein extraction. Ten embryos with radicles were ground in liquid nitrogen in chilled mortars and pestles to a fine powder. The powder was mixed with 10 ml of chilled Tris-HCl 50 mM pH 7, 2.5 mM EDTA and contained one tablet of protease inhibitor cocktail (Complete, Roche Diagnostics, Laval, Québec, Canada). Each sample was sonicated at 100 watts (25% of amplitude) three times for 15 seconds each on ice. Samples were centrifuged at 100 000 g at 4°C ± 1 for 20 minutes. The supernatant was collected and aliquoted in 1.5 ml tubes. Samples were centrifuged for one minute at 14 000 g to remove insoluble matter and the supernatant transferred to a clean 1.5 ml tube and stored at -20°C.

3.2 Determination of protein concentration

Protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad). Each protein sample was diluted to a final concentration of 1/10 and 1/100 in 50 mM Tris-HCl pH 8.0. 40 µl of Dye Reagent Concentrate (Bio-Rad) were added to each dilution. Dilutions of protein standards containing 0.02 to 1.0 mg/ml of protein were similarly prepared with bovine serum albumin solution (BSA) (BD Biosciences, CA, USA). Absorbance at 595 nm was measured in a plate reader (Synergy HT BIO-TEK Instruments, Inc., Winooski, Vermont, USA). A standard curve was made by simple linear regression using the values obtained with the BSA dilutions and the concentration of the protein samples was determined by interpolation.

3.3 SDS-PAGE of soybean proteins

SDS-PAGE was used to compare the protein profile between water and LCO-treated seeds. Forty-five μg of protein samples were mixed with 4X loading dye (10% SDS, 20% glycerol, 0.5 M Tris-HCL pH 7.5, 10% β-mercaptoethanol, 0.5% (W/V) Bromophenol blue), boiled for 5 minutes and kept on ice until loading. A 12.5% polyacrylamide separating gel (0.375 M Tris-HCl pH 8.8, 0.1% SDS, 12.5% bis-acrylamide, 0.05% ammonium persulfate, 0.05% TEMED) with a 4% stacking gel (0.125 M Tris-HCl pH 6.8, 0.1% SDS, 4% bis-acrylamide, 0.05% ammonium persulfate, 0.1% TEMED) were prepared. Samples were run with the Protean II xi cell (Bio-Rad) filled with 800 ml of running buffer (25 mM Tris-HCl pH 8.8, 192 mM glycine, 0.1% SDS) for 1hrs 30 at 13 mA followed by 5hrs at 18 mA. Gels were stained with a Coomassie blue solution (sensitivity of 300-1000 ng/band) (10% acetic acid, 25% isopropanol, 0.025% Coomassie blue R-250) and washed with a destaining solution (45% methanol, 10% acetic acid). The molecular weight of proteins was evaluated using BenchMark prestained protein ladder (Invitrogen) ran in the same gel.

RESULTS AND DISCUSSION

1. Changes induced in N. tabacum cv. Xanthii *following LCO treatment*

1.1 Photosynthesis analysis of tobacco sprayed with LCO

It was demonstrated that LCO sprayed on leaves increased the photosynthetic rate of plants from different botanical families such as soybean, corn, rice, melon, canola, apple and grape (Donald L. Smith, personal communication; Khan et al., in press). The same group also showed that LCO-treated plants had more leaf area and dry weight compared to controls. Following these observations, different experiments were conducted using tobacco to determine whether this species responded to LCO treatment.

The first aspect that was studied was the photosynthetic rate. Leaves were sprayed until saturation with water or purified LCO. Photosynthesis was measured at the same time of day under greenhouse conditions. Figure 3 presents the photosynthetic rate measured on the same leaf for a period of ten days for tobacco treated with water or 10^{-8} M of LCO. A variation in photosynthetic rate was observed between days. No significant difference was evident between the two treatments at the same day collected and no trend over time was noticed. Concentrations of 10^{-6} and 10^{-10} M of LCO were also used and similarly, no significant difference between the two treatments was observed (data not shown).

A similar experiment was performed in parallel, but the samples were harvested and frozen every day for future analysis (results not shown). At each day, the photosynthesis analysis was conducted on different plants. Four replicates each were

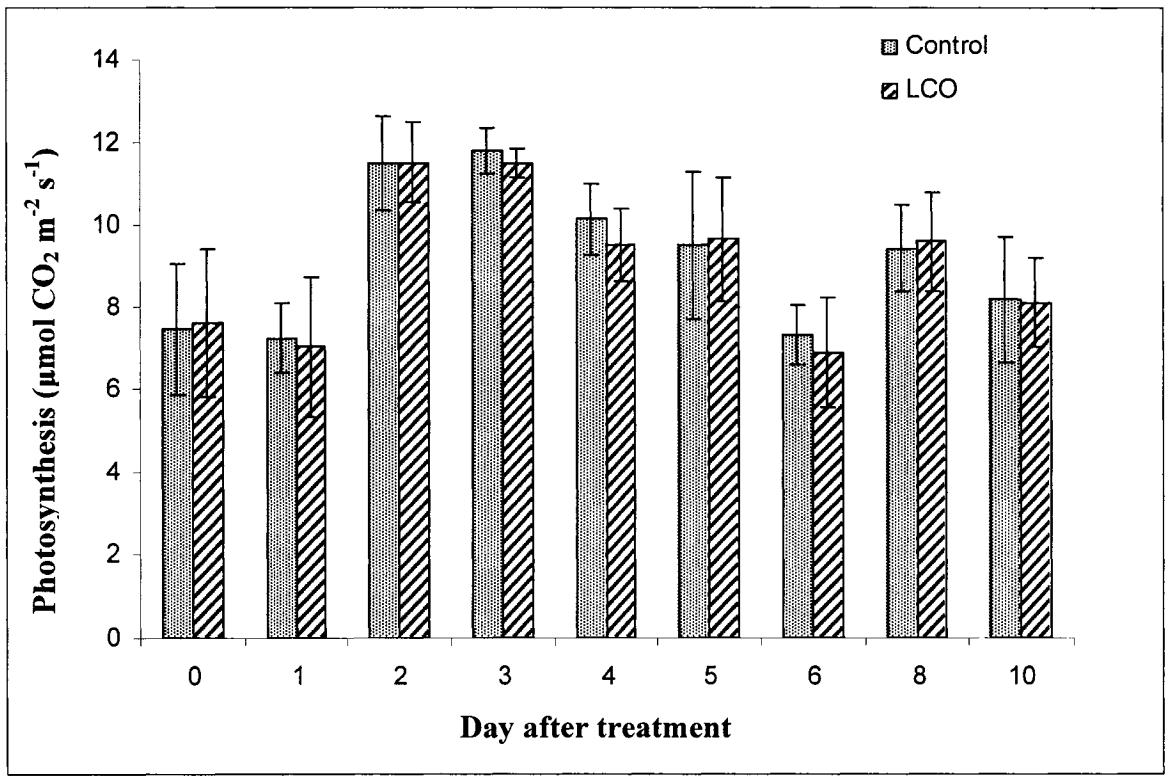


Figure 3. Photosynthesis measured in tobacco treated with water (control) or 10^{-8} M of LCO. Photosynthesis analysis was performed in *N. tabacum* cv. Xanthii sprayed until saturation with control (water) or LCO 10^{-8} M. Measurements were conducted in the greenhouse at the same time during the day and the same leaf was chosen every day. Each bar represents the average of six replicates ± the standard error. One-way analysis of variance (ANOVA) was used to assess statistical differences ($P<0.05$) and the least significant difference (LSD) method was used to determine significant differences among means.

done for water and LCO 10^{-8} M treatments. We observed that the control was sometimes higher than the LCO treatment, and sometimes lower. On the sixth day, a statistically significant increase of 5% in photosynthesis for LCO-treated plants was observed. This increase was initially expected to appear earlier and was also anticipated to be over a period of at least two days. In previous experiments performed by Khan et al. (in press), the photosynthetic rate for the LCO treatment increased from day 1 to day 4, followed by a decrease. The LCO effect on photosynthesis was on day 4. On day 5, the photosynthetic rate for both LCO and water control treatments was similar (Khan et al., in press). An increase of more than 5% was also expected. 10-36% increase in photosynthesis was observed on legumes and non-legumes after LCO application (Donald L. Smith, personal communication; Khan et al., in press). Therefore, the 5% increase observed on the sixth day appeared to be aberrant since it was not reproduced in other similar experiment and that there was no similarity with the previous works performed by Smith (personal communication) and Khan et al. (in press).

In most of the experiments described by Smith (personal communication) and Khan et al. (in press), the largest increase was detected on day 4. It was surprising that tobacco did not give a clear response to the LCO treatment because it was found in the past that LCOs affected tobacco protoplasts (Rohrig et al., 1996; Rohrig et al., 1996; Rohrig et al., 1995). However, the LCOs used in these studies were synthetic, which are different from the LCO used in our work. The LCO stock used to carry out the experiment was not likely the source of the problem since it was tested previously to be biologically active using a soybean seed germination test as shown in Figure 4: the LCO lot was considered active if it stimulated soybean seed germination (compared to the water control). The increase in seed germination following LCO treatment was significant ($P<0.05$) compared to the control for each of the three independent experiments.

For photosynthesis analysis, plants were selected at the beginning of the treatment. These plants were uniform and of the same height. In experiments done by others, an increase in photosynthetic rate for LCO-treated plants was detected with

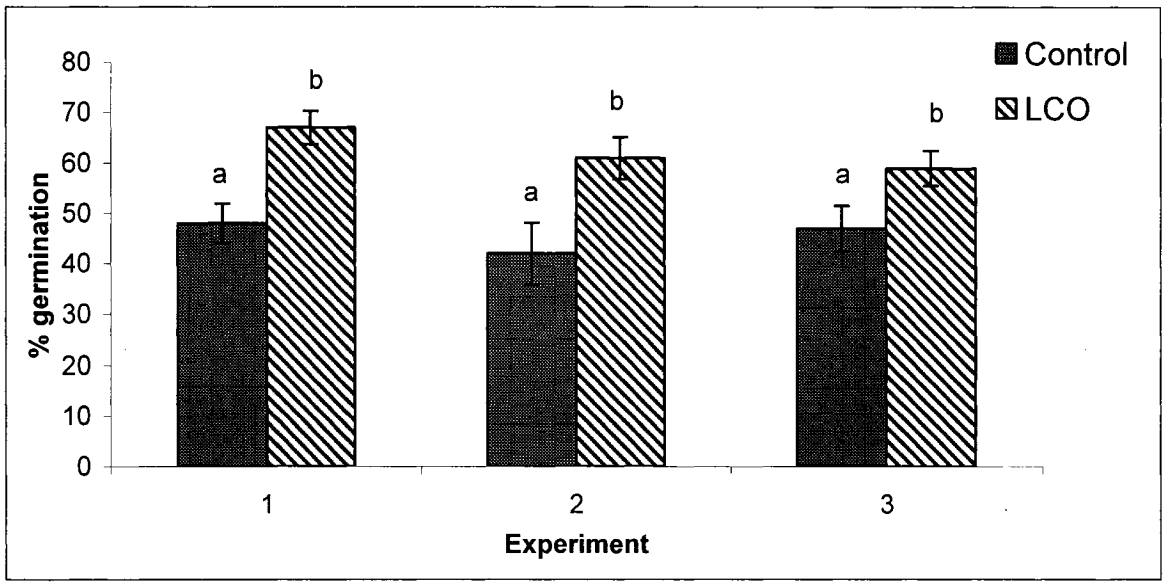


Figure 4. Results of three different germination tests on *G. max* with control (water) and LCO 10^{-8} M treatment to verify the biological activity of LCO. The observation of germination was done between 22-23hrs after the addition of water or LCO. Germination was at 24°C ± 1 for each experiment, in the dark. Each bar represents the mean of ten replicates ± standard error. Each replicate had ten soybean seeds. One-way analysis of variance (ANOVA) was used to detect statistical differences ($P<0.05$) and the least significant difference (LSD) method was used to test for significant differences among means. For the same experiment, a different letter means that a significant difference was found between the two treatments.

concentrations ranging from 10^{-6} to 10^{-12} M of LCO under greenhouse conditions (Donald L. Smith, personal communication; Khan et al., in press). This observation led us to believe that at least one of the concentrations tested (which were 10^{-6}, 10^{-8} and 10^{-10} M of LCO), should have induced an increase in photosynthetic rate compared to the control. Studies conducted by Smith (personal communication) and Khan et al. (in press) were performed with LCO dissolved in 50:50 acetonitrile/water solution. In this present study, LCO was dissolved in water. However, other studies also showed no increase of photosynthesis when soybean plants were sprayed with 10^{-8} M LCO (Cholewa et al., 2003). That group also found that under low light intensities, a decrease in photosynthesis after LCO treatment was observed compared to the control. From these results, Cholewa et al. suggested a dual role for LCO in photosynthesis, causing increases of photosynthesis at high light intensities and decreases in photosynthetic rate at low light intensities. Rhizobia is known to act only on roots starved for nitrogen and high photosynthetic activity is required to support the nodulation and nitrogen fixation processes (Bauer et al., 1996; Imsande, 1988). In our studies, the plants were fertilized every week with a solution of 3g/L of 20-20-20 K/P/N, the same treatment as in the studies conducted previously (Donald L. Smith, personal communication; Khan et al., in press). We speculate that an increase in photosynthetic rate by LCO treatment could be more accentuated if the plants were grown under nitrogen starvation.

1.2 Leaf area and dry weight analysis on tobacco treated with LCO

Leaf area and dry weight were also studied in tobacco. The results obtained for tobacco leaf area and dry weight after foliar application of water or 10^{-8} M of LCO are presented in Table 3. The total leaf area was calculated by pooling the leaf area for all leaves on the same plant. As shown in this table, the average leaf area was 1328 cm^2 ± 48.5 for control compared to 1316 cm^2 ± 63.9 for the LCO treated-plants. The results show slight decrease in total leaf area with the 10^{-8} M of LCO compared to the control. However, the variation observed was not statistically different. The increase in leaf area after LCO treatment compared to the control was expected as this is consistent with an increase of 16% previously observed by Khan et al. (in press) in corn. The average dry

Table 3. Leaf area and dry weight of *N. tabacum* cv. *Xanthii* after 15 days of treatment with LCO 10^{-8} M or water.

	Control	LCO 10^{-8} M
Average total leaf area (cm^2)[1]	1328	1316
Standard error	48.5	63.9
Average dry weight (g)[1]	5.48	5.49
Standard error	0.23	0.37

[1] Average of six replicates

weight shown in Table 3 was 5.48g ± 0.23 and 5.49g ± 0.37 for the control and LCO 10^{-8} M treated samples, respectively. No significant difference was observed between the two treatments. An increase in tobacco dry weight was also expected since a 10% increase was observed previously in corn (Khan et al., in press). As discussed in the previous section, the LCO lot used to conduct these experiments was biologically active and the plants were selected for uniformity in height before the foliar application of LCO.

The results obtained did not show a statistically significant difference in tobacco between LCO and water control treatments for photosynthetic rate, total leaf area and dry weight in tobacco. Gene expression was not measured because no statistically significant phenotypic changes were observed after LCO treatment. However, previous studies showed phenotypic changes in diverse botanical families after LCO application (Prithiviraj et al., 2003; Souleimanov et al., 2002). These studies on the phenotypic effect of LCO in host and non-host plants were conducted with LCO dissolved in 50:50 acetonitrile/water solution (Prithiviraj et al., 2003; Souleimanov et al., 2002). However, this group discovered that acetonitrile itself was producing phenotypic changes in plants in the same way as LCO (Donald L. Smith, personal communication). In our experiments, stock solutions were made by dissolving LCO in water only in order to avoid this problem. We speculate that the use of water only instead of a mix of acetonitrile and water could have affected the phenotypic response to LCO observed compared to the observations made in preceding studies. Previous studies were conducted with a batch of LCO that was not as pure as the one we used in our work (Donald L. Smith, personal communication). The purification of peak B of Nod *Bj*-V (18:1), MeFuc (used in our research) using the HPLC was made difficult by contamination from other LCO peaks also produced by *B. japonicum* (Donald L. Smith, personal communication). We speculate that this mixture of LCOs could have led to a different phenotypic response in plants after LCO treatment. During the experiments conducted on tobacco, it was sometimes difficult to control insect populations in greenhouse.

It has been demonstrated that LCOs are able to activate defense-related enzymes (Inui et al., 1997) and elicit an increase in cytosolic calcium of soybean cells similar to the response elicited by un-derivatized chitin oligomers (Müller et al., 2002). It was speculated that LCOs may work by activating a general defense response that could also be activated by insects. Therefore, the presence of insects during the assays could have stimulated the defense response of plants before treatment application and then affected the plant response to LCO. We decided to pursue the studies of the effect of LCO in *G. max* (L.) Merr cv. OAC Bayfield, which is a natural host of *B. japonicum*. Gene expression and protein profiling studies were conducted with soybean seeds treated or not with LCO.

2. *Changes induced in* G. max *(L.) Merr] cv. OAC Bayfield following LCO treatment*

2.1 Impact of LCO application on soybean seed germination

It was previously shown that LCO enhanced germination of soybean (Prithiviraj et al., 2003). These authors hypothesized that the increase in germination rate may be attributed to an enhancement of the cell cycle rate resulting from Nod factor addition. An example of stimulation of seed germination by LCO treatment for three independent experiments is presented in Figure 5. For the three experiments performed on *G. max*, the percentage of seed germination was higher with the LCO treatment than with the water control and the increase was statistically significant. The stimulation of soybean seed germination was used in this study as a preliminary test to check whether these were a physiological response to LCO. This test was performed prior to undertaking every new experiment. The enhancement of soybean germination by LCO was constant and reliable. The gene expression study following LCO treatment was performed on these three independent experiments and the results are shown in Figure 5. The quality of the synthesized cDNA was first verified by PCR in order to test for DNA contamination.

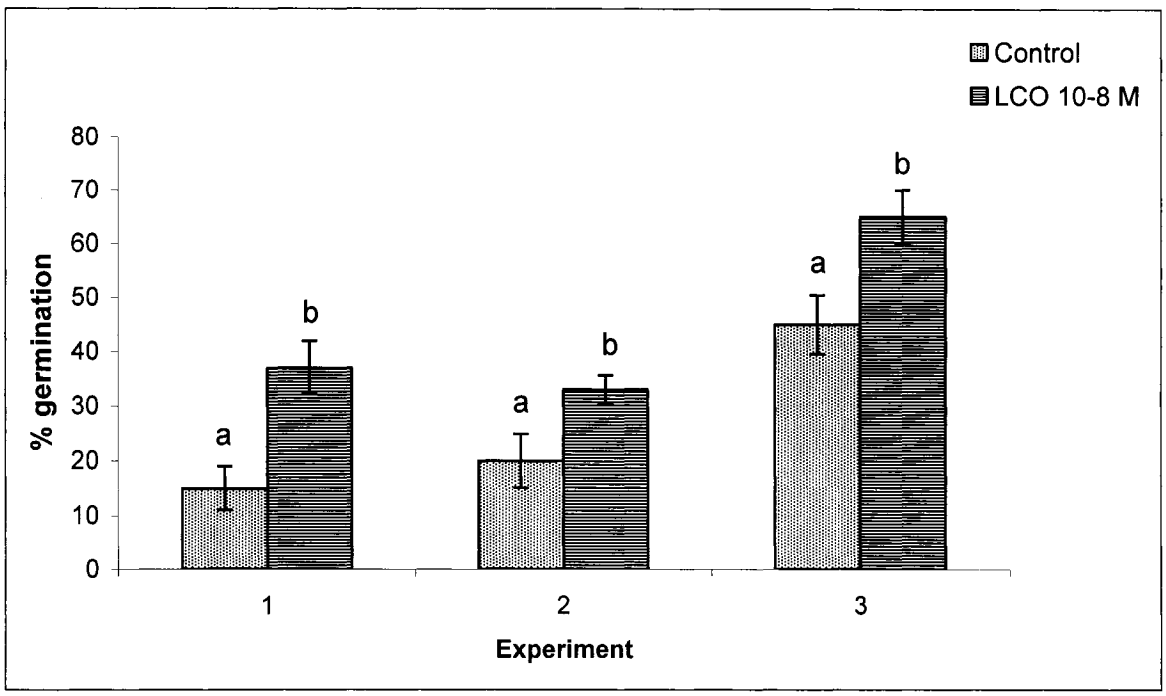


Figure 5. Seed germination results for three different experiments on *G. max* treated with 10^{-8} M LCO or water (control). These experiences were done to study gene expression on germinating seeds. The observation of germination was done between 12-13hrs 30 after the addition of water or LCO. Germination was at 24°C ± 1, in the dark. Each bar represents the mean of ten replicates ± standard error. Each replicate had ten soybean seeds. One-way analysis of variance (ANOVA) was used to assess statistical differences ($P<0.05$) and the least significant difference (LSD) method was used to test for significant differences among means. For the same experiment, a different letter denotes a significant difference found between the two treatments.

2.2 Verification of the quality of cDNA and DNA contamination

Each cDNA sample was tested for DNA contamination before gene expression analysis. This was necessary because the primer may be able to anneal to contaminating DNA and amplify it therefore distorting gene expression results. The DNAcontamination verification consisted of a PCR done with a negative control (all the components for the cDNA synthesis reaction except reverse transcriptase) for each sample. DNA contamination was detected on an agarose gel. Samples that contained DNA were treated a second time with DNase to remove contaminating DNA. Figure 6 presents an example of PCR products using the *RPL13* primer set on soybean embryo cDNA collected during experiment #1. The length of the expected fragment was 211 bp. As seen on gel **A,** PCR products of the cDNA displayed a clear band at around 220 bp. On gel **B** (corresponding to the negative control of the samples amplified in gel **A**), no band was observed, indicating that no DNA contamination was present in the samples. Each sample was examined with all the primers that were used in the study including the two internal references. After verification, gene expression was measured on the cDNA and analyzed by three different methods.

2.3 Real-time RT-PCR used to measure soybean seed gene expression

Real-time PCR detection is based on the measurement of fluorescence during PCR. The amount of fluorescence emitted is proportional to the quantity of PCR product and allows the monitoring of the PCR reaction (Klein, 2002). The RT-PCR method is more sensitive and more flexible than the other RNA quantification methods such as Northern blotting, *in situ* hybridization or RNAse protection assays (Wang and Brown, 1999). It can be used to compare the levels of mRNAs in different samples, characterize patterns of mRNA expression, discriminate between closely related mRNAs and analyze RNA structure (Bustin, 2000). However, specific and nonspecific PCR products are both detected by using DNA-binding dyes (Klein, 2002) as SYBR Green I. Primer dimers

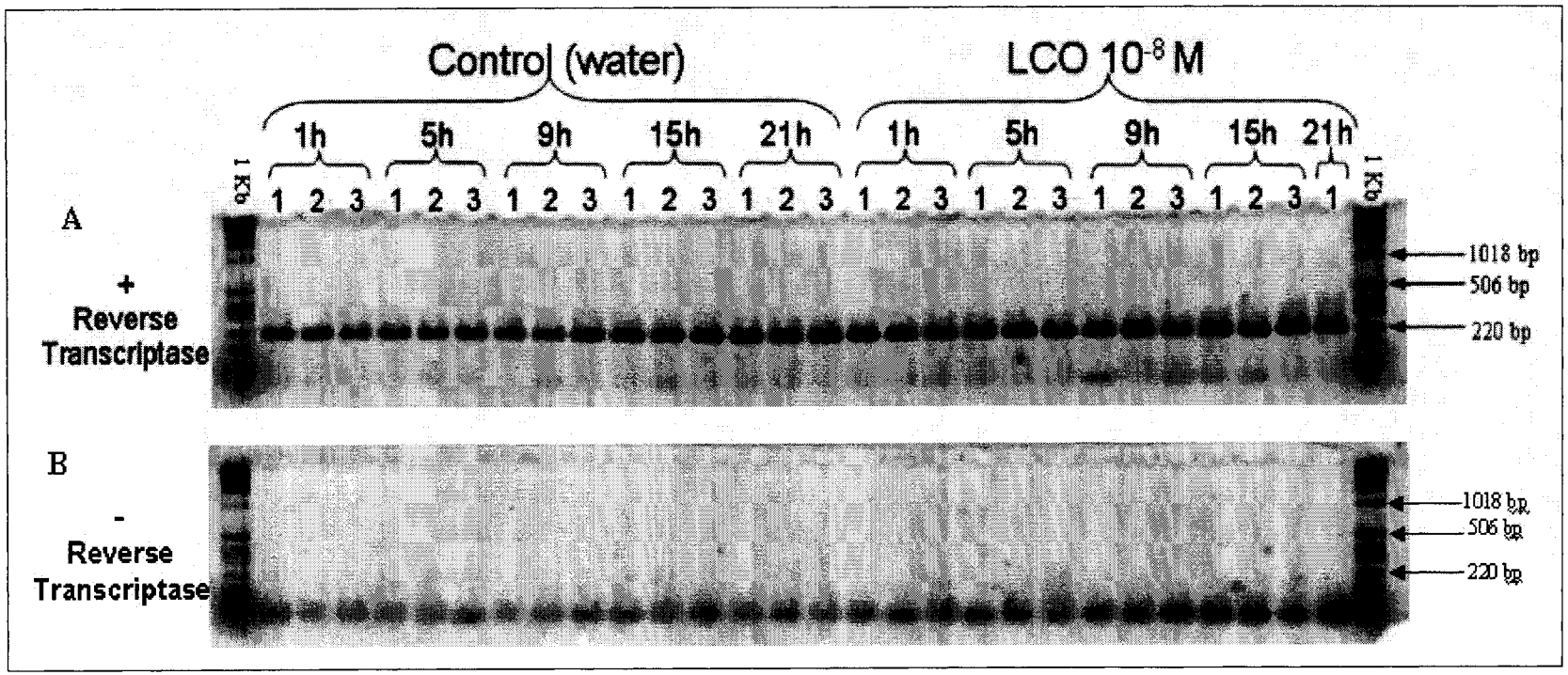


Figure 6. PCR products amplified using the *RPL13* primer set on cDNA from *G. max* treated with 10^{-8} M LCO or water (control) to check for possible DNA contamination. **A)** PCR products made on cDNA. The length of the expected fragment was 211 bp. The numbers above the wells represent the replicates (containing a pool of ten seeds) for each time point collected from control and LCO treatments. Each treatment had five time points: 1, 5, 9, 15 and 21hrs. **B)** Negative control (all the components for the cDNA synthesis reaction except reverse transcriptase) of the above corresponding samples.

formed during PCR are the products of nonspecific elongation of PCR primers (Ball et al., 2003) and compete with generation of a specific PCR product, leading to reduced amplification efficiency of PCR (Roche Molecular Biochemicals, 1999). These artefacts can be reduced by careful primer design, optimization of the PCR with "hot-start" PCR and amplification under stringent conditions (Harris and Jones, 1997). Ball et al. (2003) also suggested the modification of the real-time RT-PCR (LightCycler) strategy to overcome this problem by adding an additional segment after the elongation. This step allows the measurement of fluorescence at a temperature greater than the melting point of primer dimers but lower than the melting temperature of the desired product. For example, the fluorescence determination after each cycle for the specific RPL13 products was performed at 78°C instead of 72°C in order to circumvent primer dimer interference.

The melting curve analysis was performed on the reaction to identify LightCycler products generated in the presence of SYBR Green I. This analysis allows differentiation of both desired PCR products from primer dimers by their characteristic melting behavior (Roche Molecular Biochemicals, 1999). Figure 7A shows a melting temperature profile specific to the *RPL13* primer set generated using cDNA from soybean seed samples [the graph shows the rate of change in fluorescence (-dF1/dT) as a function of temperature]. The product of 211 bp displays a characteristic melting temperature at 82°C. No primer dimer formation is observed on this graph. Figure 7B presents the LightCycler product accumulation for the samples used to perform the melting curve analysis in Figure 7A. Electrophoresis is not required as evidence of presence of product since the melting curve analysis and the product accumulation graphs show product amplification for each sample. However, LightCycler amplification products were electrophoresed in order to confirm that the characteristic melting temperature was associated with the size of amplicon expected with a specific set of primer.

Figure 8 presents real-time RT-PCR products performed with soybean seed cDNA collected in experiment #1. Figure 8A shows amplification products generated using the *RPL13* primer set corresponding to the samples used in the melting curve analysis and

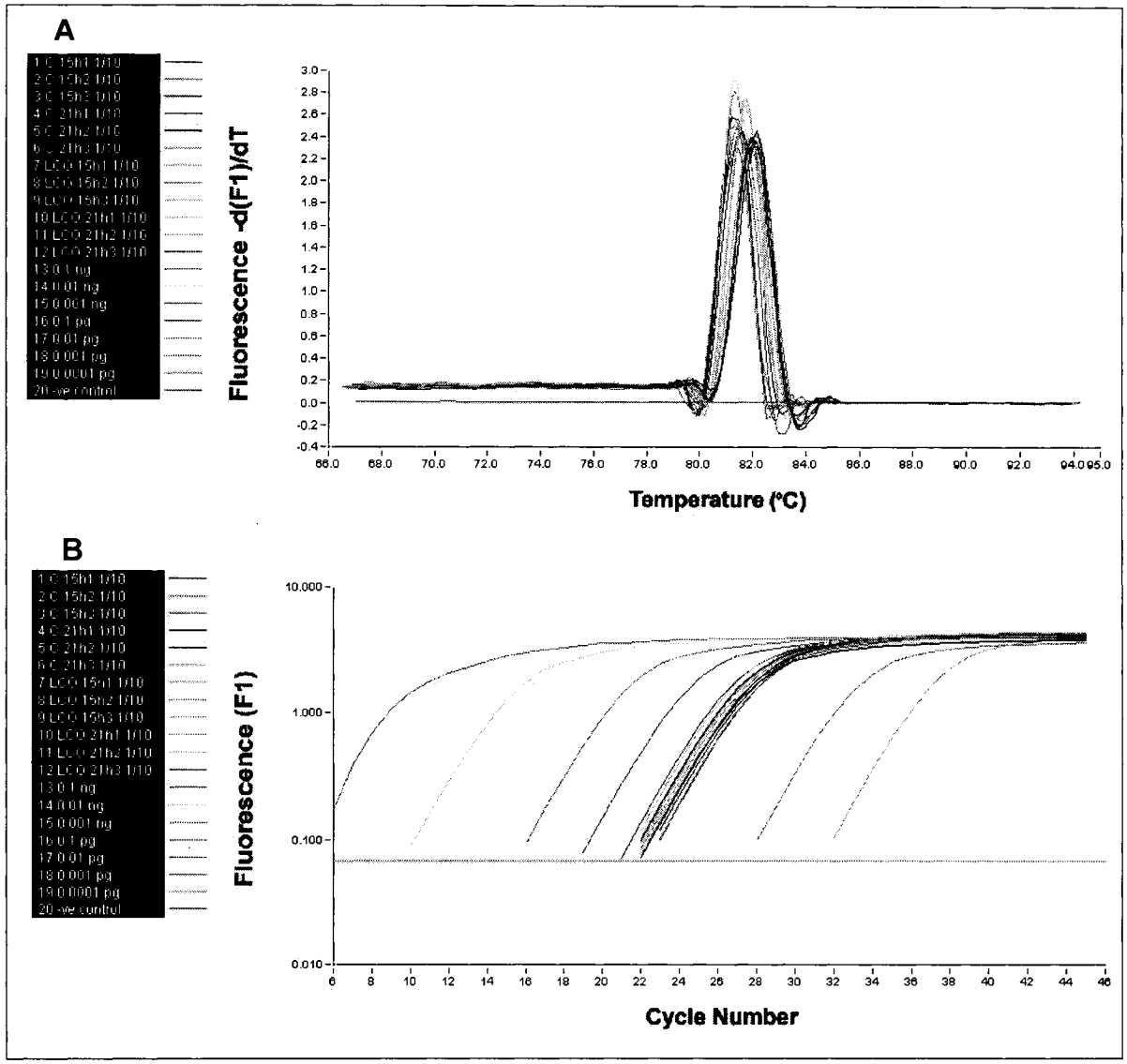


Figure 7. cDNA from *G. max* treated with 10^{-8} M LCO or water (control) collected in experiment #1 and amplified with the *RPL13* primer set using real-time PCR (LightCycler). **A)** Samples analyzed generated a melting temperature profile specific to the *RPL13* primer set used with soybean. The product of 211 bp displays a characteristic melting temperature of 82°C. **B)** Sample amplification is visualized and the crossing point (CP) determined: the CP is the cycle at which PCR products begin to increase exponentially.

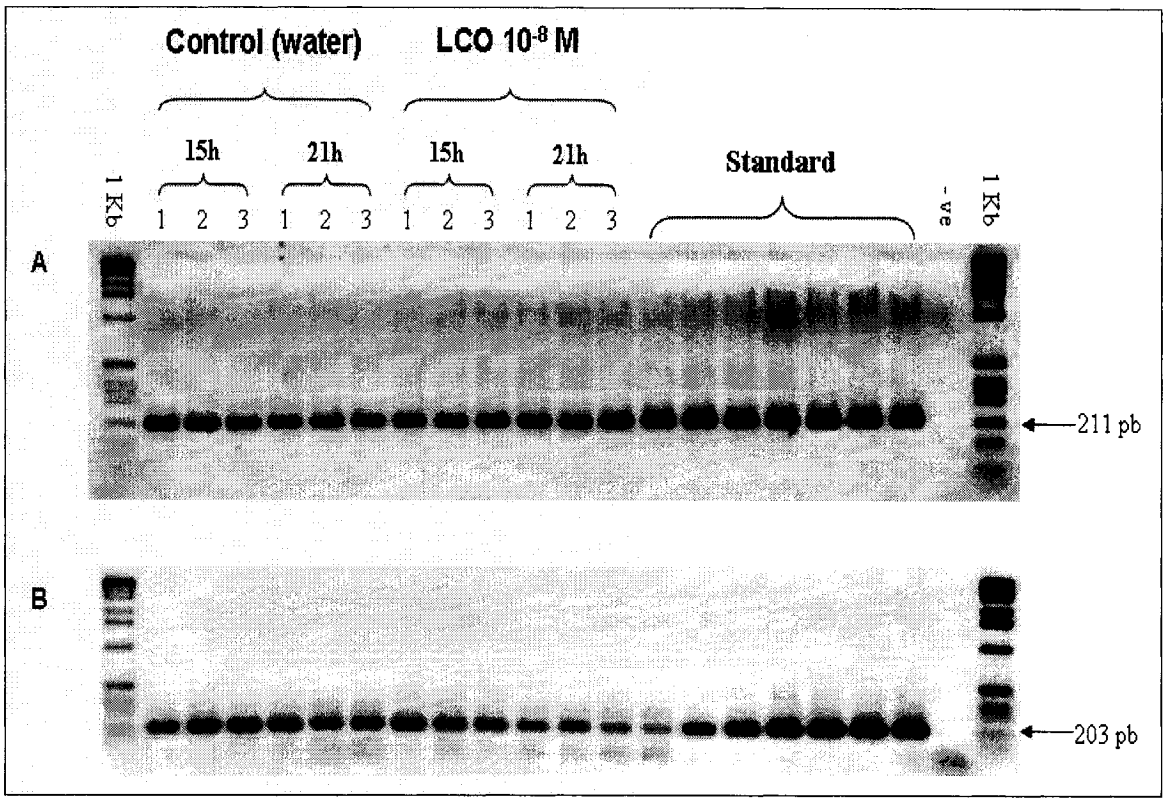


Figure 8. Agarose gel of real-time PCR products performed with cDNA form *G. max* seed treated with 10^{-8} M LCO or water (control) from experiment #1. **A)** cDNA samples were amplified with the *RPL13* primer sets. The melting peak corresponding to these samples was presented in Figure 7A. The size of the expected product was 211 bp. **B)** cDNA samples amplified using the *ICL1* primer set. The size of the expected product was 203 bp. The negative (-ve) control was made with DDH_2O instead of cDNA in both cases.

product amplification graph of Figure 7. The base pair size of expected amplicons was 211 bp and no amplification product was detected in the negative control. The melting temperature at 82°C corresponded to the 211 bp product expected with the *RPL13* primer set. Figure 8B shows another example of real-time RT-PCR products performed using the *ICL1* primer set. The expected product size was 203 bp and no amplification product in the negative control was detected either on the agarose gel or the melting curve profile (data not shown).

The RT-PCR reaction is characterized by significant variation and non-reproducibility, even with identical samples between different laboratories (Freeman et al., 1999; Keilholz et al., 1998). Its reproducibility is potentially compromised by the variable efficiency of the RT itself and by the need for two sequential enzymatic steps (Bishop et al., 1997). The reproducibility of the RT-PCR reaction was monitored in this study. Intra-assay precision was investigated using four different samples in three repeats within one LightCycler reaction (Pfaffl, 2001). Inter-assay variation was determined in three different experimental reactions performed on three days (Pfaffl, 2001). Within the same LightCycler reaction, the standard error for CP data between the triplicates was, on average, 0.75% with a maximum of 2% and a minimum of 0.16% (data not shown), better than the 14% reported for conventional RT-PCR (Zhang et al., 1997). The variability between assays of samples performed in triplicate ranged from 0.12 to 0.37%. These results therefore showed minimal variation within the same reaction and between reactions, increasing the confidence in the gene expression results.

Three different experiments were conducted in order to study gene expression of *cdc2*, *WASI*, *ICL1* and *14-3-3* genes after LCO treatment. The first experiment had five time points (1, 5, 9, 15 and 21hrs after the addition of treatment solution) with ten replicates per treatment. A possible increase in gene expression with LCO treatment was observed with the gene coding for isocitrate lyase at 9hrs following the addition of LCO as shown in Figure 14. A second and third experiment was run using the same conditions, but with more time points around 9hrs (1, 5, 7, 9, 11, 15hrs after the addition of each treatment) to focus on the possible response.

2.4 Comparison of three different methods to analyze gene expression in soybean following LCO application

The first method used to analyze gene expression results compared the crossing point (CP) of the control and the LCO 10^{-8} M treatment. The CP is the cycle at which PCR amplification begins to show an exponential growth phase and is considered to be the most reliable point that is proportional to the initial concentration (Roche Molecular Biochemicals, 2001). The lower the CP, the larger the initial amount of cDNA for the gene studied. The results of an experiment with both control and LCO-treated soybeans are presented in Figure 9. This method of analysis did not include a correction for the magnitude of gene expression using an internal reference. The sample capacity of the LightCycler was 32. The number of samples used to assess gene expression was 36, and two different LightCycler reactions were required to analyze all samples, making graph interpretation more difficult. In experiment #1, the first reaction included samples collected 1, 5 and 9hrs after treatment and the second reaction included samples collected 15 and 21hrs after treatment. For experiments #2 and #3, samples collected 1, 5 and 7hrs after treatment were analyzed by RT-PCR in a separate reaction from those collected at 9, 11 and 15hrs after treatment.

Figures 9 and 10 present the comparison of transcript levels for *cdc2,* the control and the 10^{-8} M LCO treatments on soybean for experiments #2 and #3. The experiments were conducted independently and analyzed using the CP comparison method. Both graphs show a decrease in CP over time. Standard errors vary between 0.03 and 0.75. These values are small, which implies that the number of replicates is sufficiently adequate to be representative. No significant difference was found between the treatments for time points 1, 5, 7, 9 and 15hrs for both experiments. However, in experiment #3, a significant increase in LCO-treated samples was observed at 11hrs after treatment. This was not observed for experiment #2 at 11hrs after LCO treatment.

Figure 11 presents the comparison of transcript levels for the *14-3-3* gene, the control and the 10^{-8} M LCO treatments on soybean for experiment #2 and analyzed using

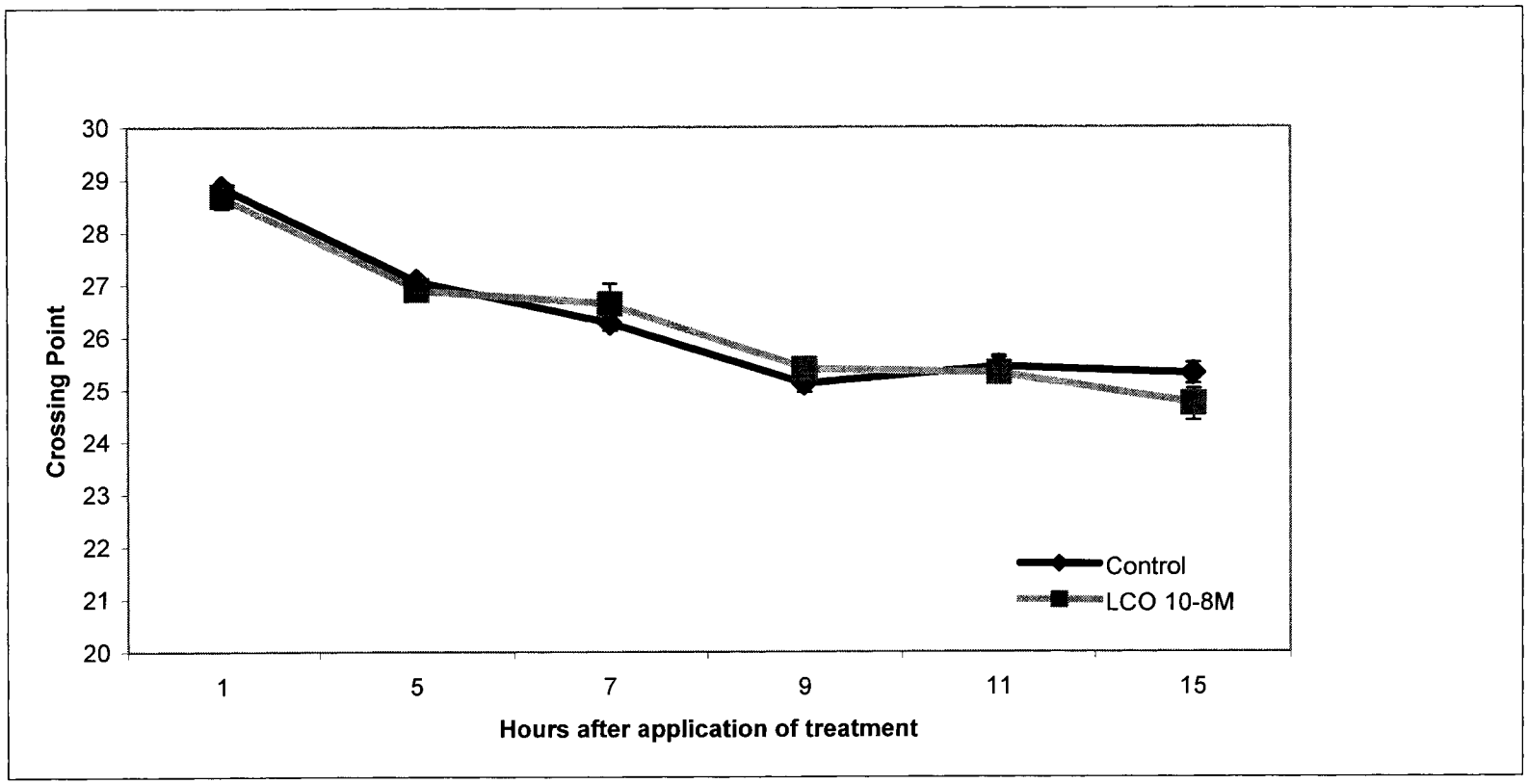


Figure 9. *cdc2* gene expression at different times after LCO treatment in soybean embryos collected in experiment #2. Each point represents the mean of three replicates ± standard error. Ten embryos per replicate were collected at different time points following the addition of LCO or control solutions. The crossing point (CP) was the cycle at which PCR products began to increase exponentially. The lower the CP, the higher the initial amount of *cdc2* cDNA present in the reaction. One-way analysis of variance (ANOVA) was used to detect statistical differences ($P<0.05$).

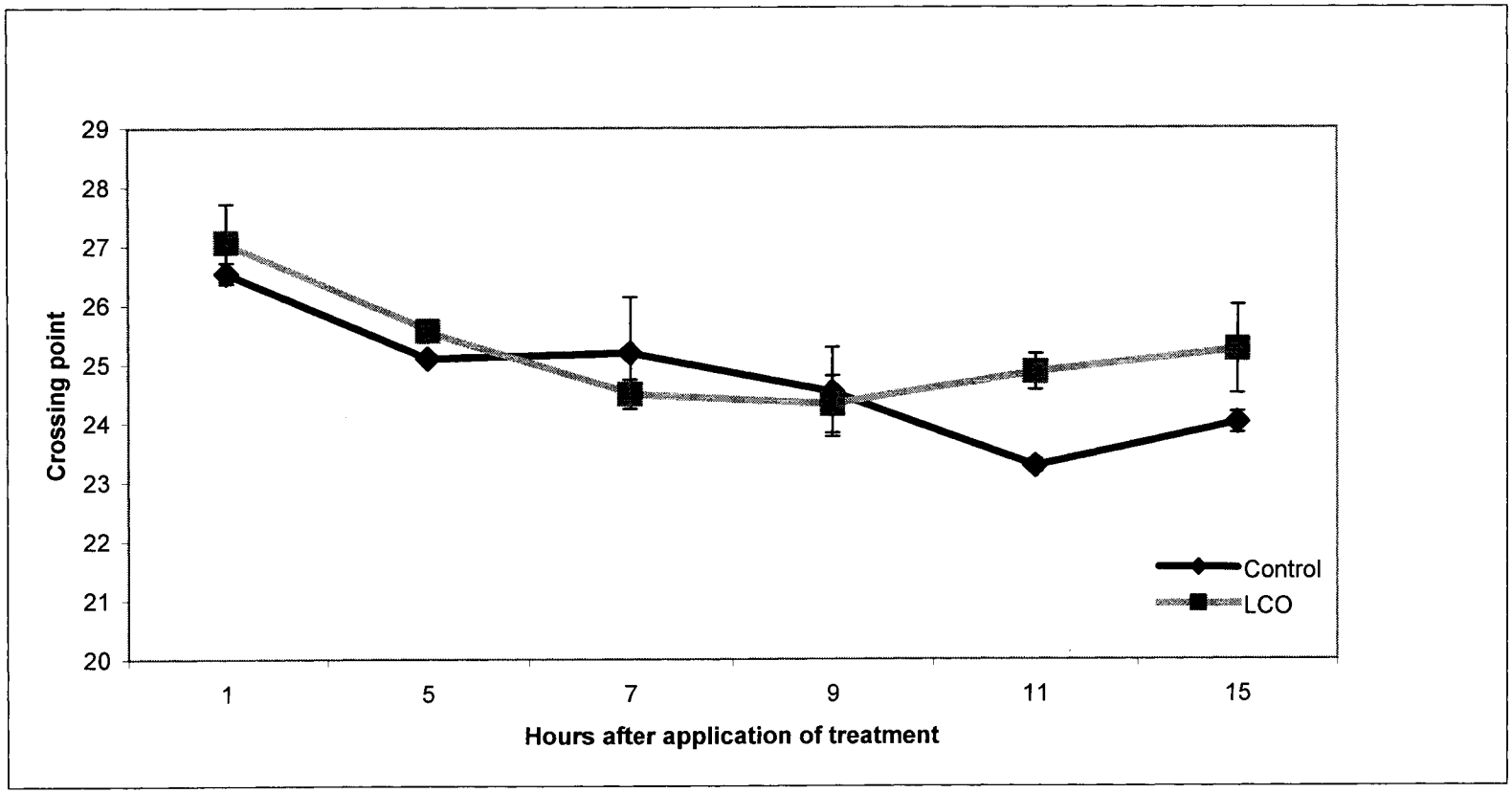


Figure 10. *cdc2* gene expression at different times after LCO treatment in soybean embryos collected in experiment #3. Each point represents the mean of three replicates ± standard error. Ten embryos per replicate were collected at different time points following the addition of LCO or control solutions. The crossing point (CP) was the cycle at which PCR products began to increase exponentially. The lower the CP, the higher the initial amount of *cdc2* cDNA present in the reaction. One-way analysis of variance (ANOVA) was used to detect statistical differences ($P<0.05$).

the CP comparison method. A decrease in CP over time was also observed with this gene. Standard errors vary between 0.08 and 0.34. These values are small which implies that the number of replicates is adequate. No significant difference was found between the two treatments at each time point for the *14-3-3* gene (Figure 111). The expression of *cdc2* and *14-3-3* genes studied was projected to increase over time during germination as shown previously (Potokina et al., 2002; Rylott et al., 2001; Testerink et al., 1999). As expected, a decrease in CP for both *cdc2* and *14-3-3* genes, indicating an increase in transcripts was observed as shown in Figures 9, 10 and 11.

In general, for the same gene, the trends were similar for the three experiments performed independently using the CP comparison method. In some cases, the three independent experiments showed slight variation in trends, but these divergences were not significantly different. One-way analysis of variance (ANOVA) was used to detect statistical differences ($P<0.05$) between each time point and the least significant difference (LSD) method was used to determine significant differences among means. No difference was observed between the control and the LCO treatment for the *WASI* (α-amylase inhibitor) and *ICL1* (isocitrate lyase) genes also studied (data not shown). As for the *cdc2* and *14-3-3* genes, *ICL1* transcript levels were expected to increase over time, as demonstrated previously in germinating seeds (Rylott et al., 2001). A decrease in CP over time was also observed here, with the *ICL1* gene indicating an increase in transcript levels after addition of LCO (data not shown). The CP of *WASI* gene increased over time, indicating a decrease in transcript levels for this gene (data not shown). These results were consistent with studies done by Potokina et al. (2002) showing a decrease in gene expression during germination.

However, this method of analysis did not include the internal reference normalization. It also compared different reactions without a comparison of PCR efficiency between samples.

The second and the third methods for analyzing gene expression results were the Pfaffl (Pfaffl, 2001) and Liu (Liu and Saint, 2002) mathematical transformations that are

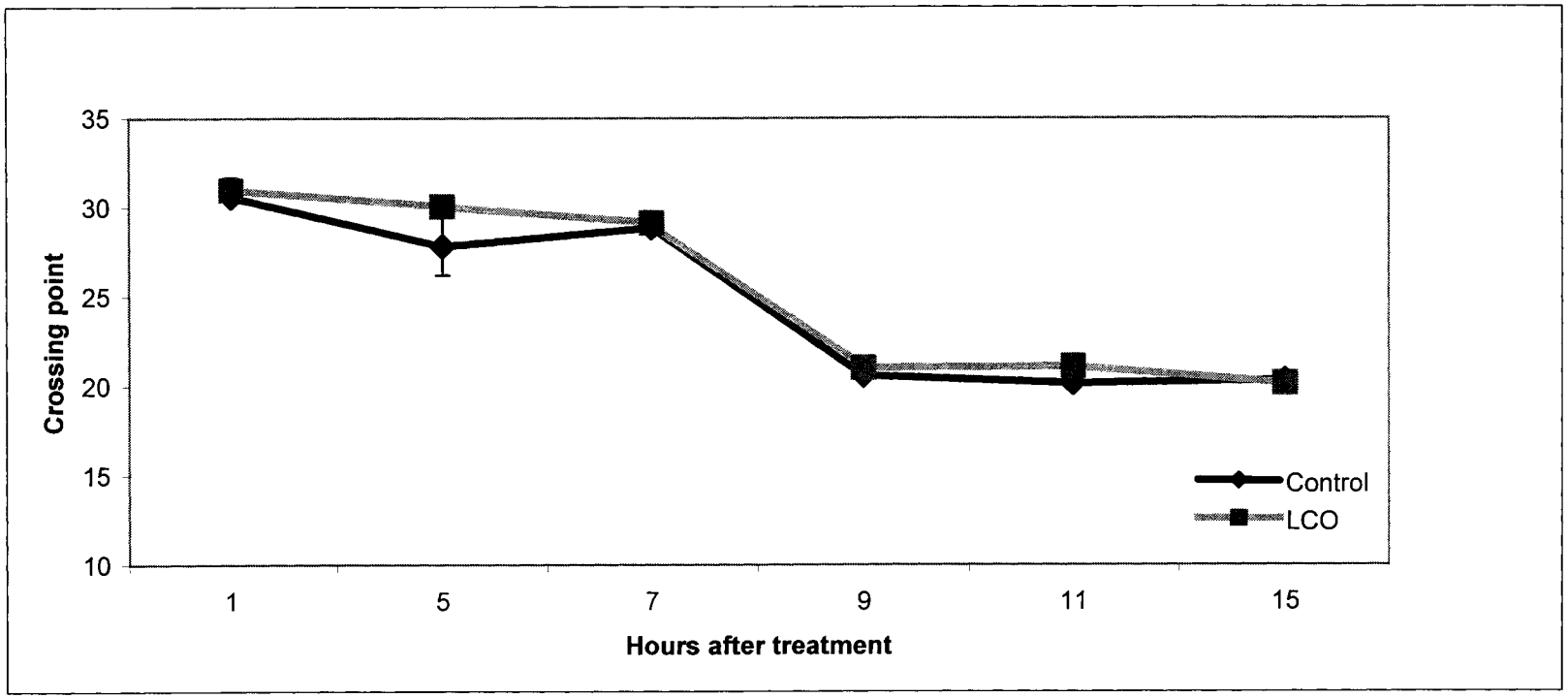


Figure 11. *14-3-3* gene expression at different times after LCO treatment in soybean embryos collected in experiment #3. Each point represents the mean of three replicates ± standard error. Ten embryos per replicate were collected at different time points following the addition of LCO or control solutions. The crossing point (CP) was the cycle at which PCR products began to increase exponentially. The lower the CP, the higher the initial amount of *14-3-3* cDNA present in the reaction. One-way analysis of variance (ANOVA) was used to detect statistical differences ($P<0.05$).

described as a relative method to compare patterns of gene expression. Both required the use of an internal reference to adjust results of gene expression between reactions and between samples (Bustin, 2002). Use of a reference gene also corrects for variation in the initial sample amount and for variation in cDNA synthesis efficiency (Roche Molecular Biochemicals, 2001). The ideal internal control should be expressed at a constant level among diverse tissues of an organism, at all stages of development, and should be unaltered by the experimental treatment (Bustin, 2000). Following the normalization, the relative gene expression results were expressed as a ratio (see section 2.6 in material and methods for formulas). A ratio of one with the mathematical method proposed by Liu indicated that the amount of transcripts from treated samples was equal to the amount of transcripts from non-treated samples (Liu and Saint, 2002). For the mathematical method proposed by Pfaffl, a ratio of zero indicated that the amount of transcripts from treated samples was equal to the amount of transcripts from non-treated samples (Pfaffl, 2001).

Recent publications have reported the possibility that expression of housekeeping genes varies considerably (Bustin, 2000; Suzuki et al., 2000; Thellin et al., 1999). Consequently, it was recommended to employ more than one internal standard and compare results to avoid errors related to the use of only one housekeeping gene (Vandesompele et al., 2002). Therefore, the expression of the housekeeping gene chosen for the experiment needs to be measured before using it as a reference (Klein, 2002). We used two housekeeping genes as internal references: *RPL13,* coding for ribosomal L13 (Vandesompele et al., 2002), and *gtub* coding for gamma-tubulin (Thellin et al., 1999). Results showing gene expression of *ICL1* following LCO treatment and normalized with *RPL13* or *gtub* by using two different mathematical transformations are presented in Figures 12, 13, 14 and 15. These examples were used to compare the two housekeeping genes used as internal references. Usually, for the same gene, samples normalized with the two different internal controls gave similar results over time and this applied for both the Pfaffl and Liu methods of calculation (Figures 12, 13, 14 and 15). Vandesompele et al. (2002) suggested a method for comparing two internal references. They calculated the ratio of the results obtained with two control genes in two different samples and termed it the single control normalization error, E. For two ideal internal reference genes (i.e. a

constant ratio between the genes in all samples), E equals 1 (Vandesompele et al., 2002). In our case, the E value calculated for the two reference genes, *RPL13* and *gtub*, ranged from 0.8 to 1.2 (data not shown). These variations were small enough to consider the use of both internal controls to normalize the data. The standard error calculated at each time point was lower using *RPL13* as an internal control compared to *gtub* in 60% of the relative gene expression study conducted on *cdc2*, *WASI*, *ICL1* and *14-3-3* genes in experiments #1, #2 and #3. Therefore, results were analyzed relative to *RPL13* gene expression.

Statistics were performed after the mathematical data transformation. Therefore, standard error was calculated on the difference between LCO and water treatments and not directly on CP values. This led to a minor increase in the standard error. Standard error was also increased through the coupling of each LCO-treated sample with its corresponding control. For example, LCO-treated replicate #1 was compared with control replicate #1 even though the comparison with LCO-treated replicate #1 might yield a higher standard error value than when compared to control replicate #2. The order in which samples were collected was respected.

Figures 12 and 13 show *ICL1* gene expression normalized relative to *gtub* or *RPL13*, respectively, using the Pfaffl method for experiment #1. In Figure 12, LCO treatment significantly increased gene expression compared to the control at the 5hrs data point in experiment #1. These results were not obtained using *gtub* for normalization with the Pfaffl method (Figure 13). Figures 14 and 15 present *ICL1* gene expression normalized relative to *gtub* or *RPL13* using the Liu method for experiment #1. These two figures show a significant decrease in *ICL1* gene expression at 1hr using the Liu method for both *RPL13* and *gtub* internal reference normalizations. However, this decrease was not noticed for this gene in experiments #2 and #3. For the other genes studied, *cdc2, 14-3-3* and *WASI,* a significant difference was occasionally observed depending on the calculation method or the internal reference used (data not shown). No significant variation of gene expression by LCO treatment at a specific data point was found in all the three independent experiments.

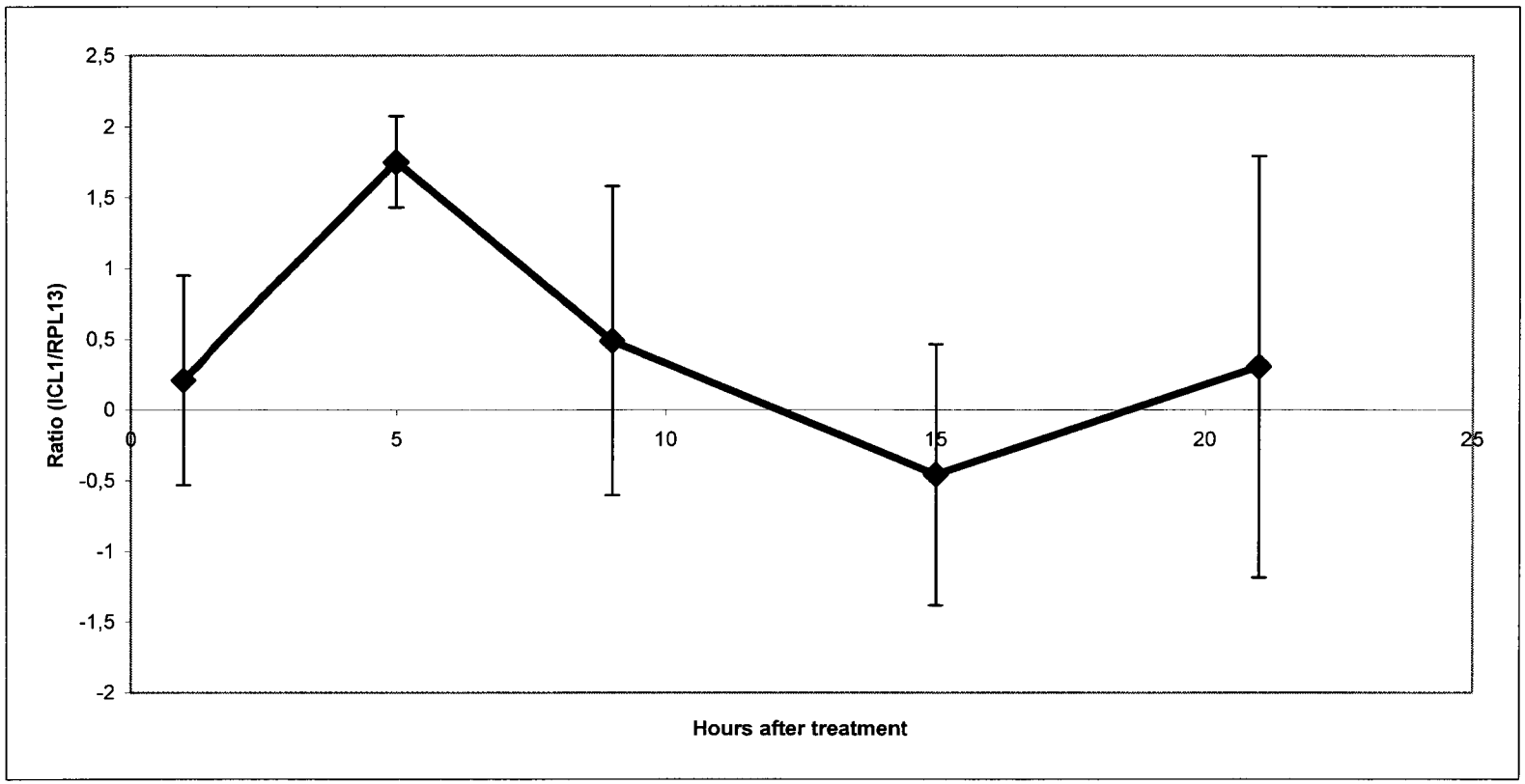


Figure 12. Soybean *ICL1* relative expression at different times after LCO treatment and normalized with *RPL13* analyzed using the Pfaffl transformation method. Each point represents the mean of three replicates ± standard error. Relative gene expression was measured in soybean embryos collected at different times after the addition of LCO or water treatment in experiment #1. A ratio of zero means that the number of *ICL1* transcripts from treated soybean was equal to the number of *ICL1* transcripts from the non-treated soybean. A two-tailed t-test was performed ($P<0.05$) to assess statistical differences.

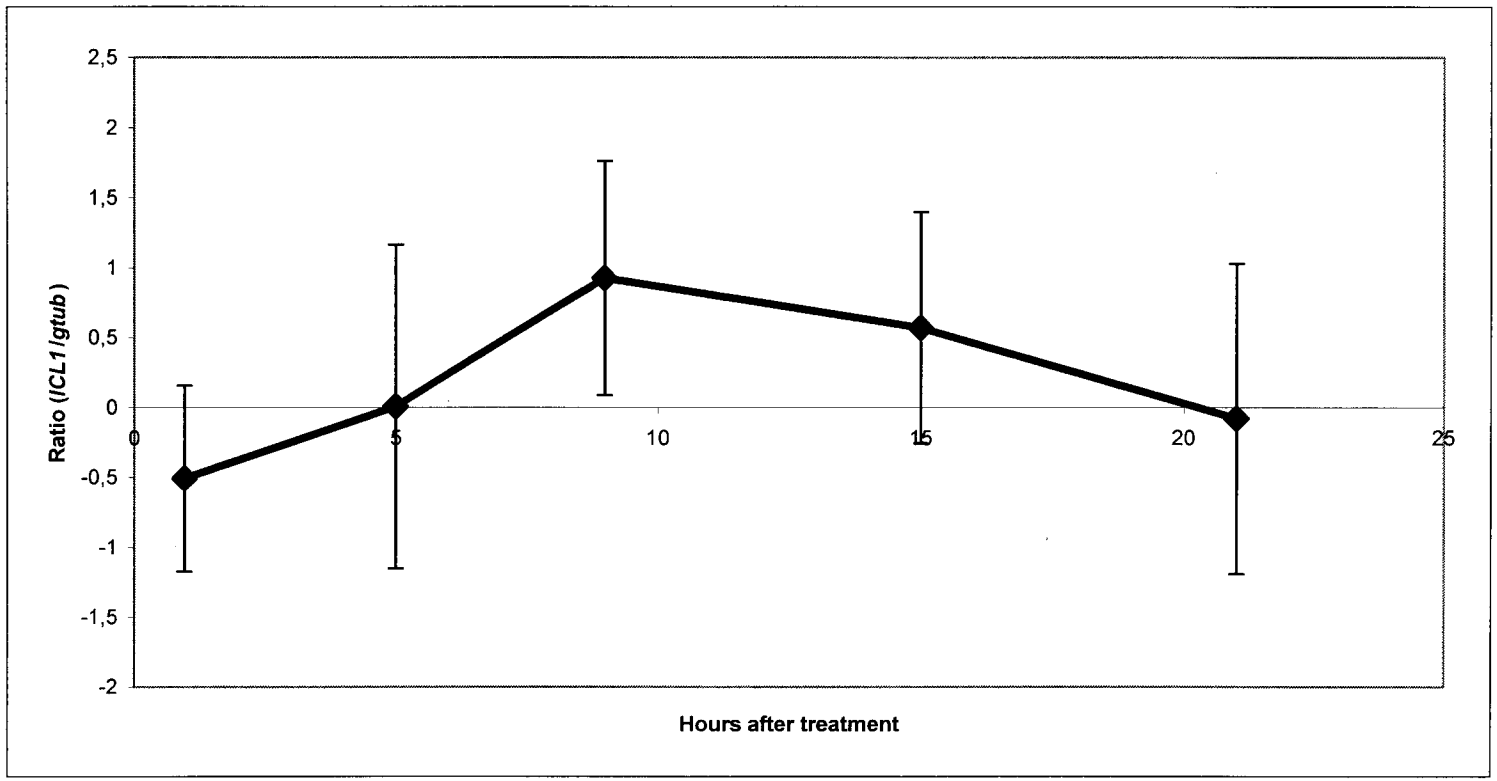


Figure 13. Soybean *ICL1* relative expression at different times after LCO treatment and normalized with *gtub* analyzed using the Pfaffl transformation method. Each point represents the mean of three replicates ± standard error. Relative gene expression was measured in soybean embryos collected at different times after the addition of LCO or water treatment in experiment #1. A ratio of zero means that the number of *ICL1* transcripts from treated soybean was equal to the number of *ICL1* transcripts from the non-treated soybean. A two-tailed t-test was performed ($P<0.05$) to assess statistical differences.

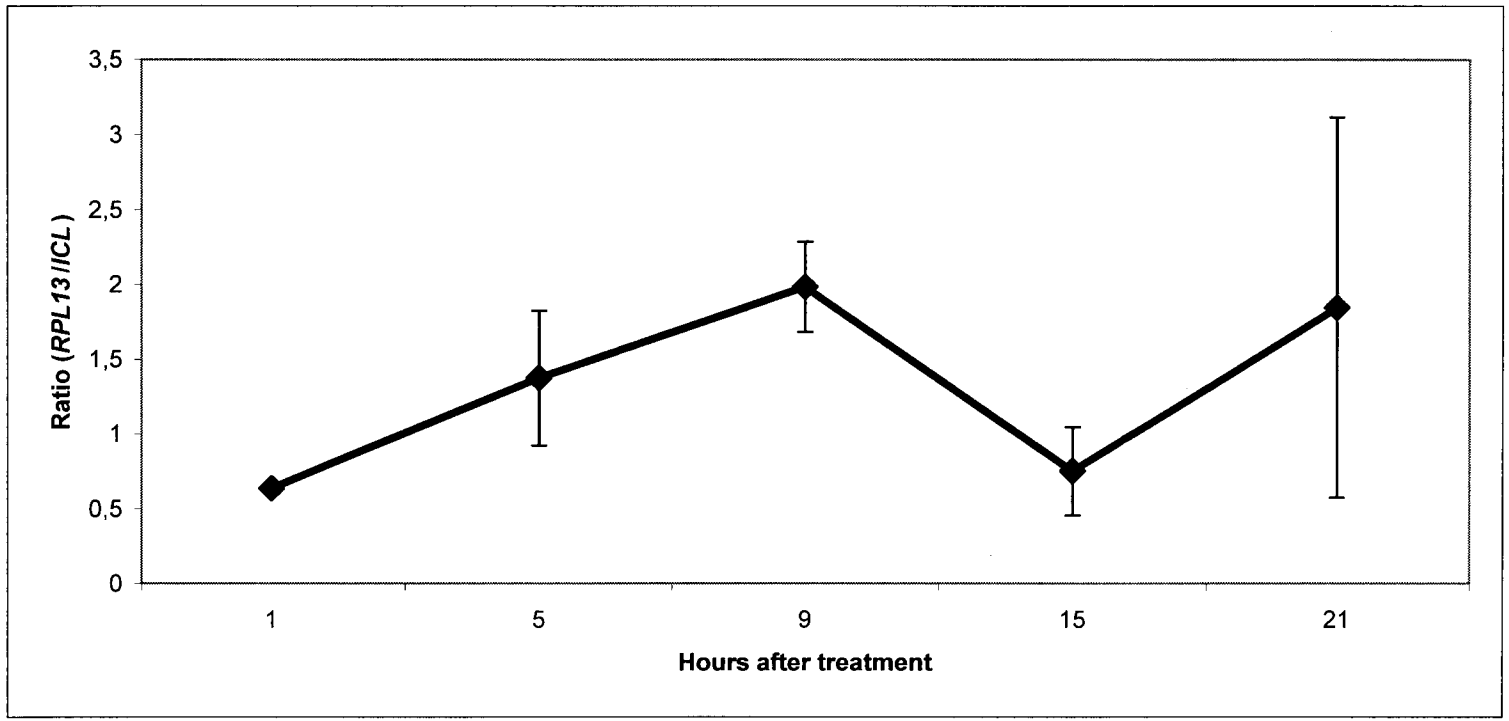


Figure 14. Soybean *ICL1* relative expression at different times after LCO treatment and normalized with *RPL13* analyzed using the Liu transformation method. Each point represents the mean of three replicates ± standard error. Relative gene expression was measured in soybean embryos collected at different times after the addition of LCO or water treatment in experiment #1. A ratio of one means that the number of *ICL1* transcripts from treated soybean was equal to the number of *ICL1* transcripts from the non-treated soybean. A two-tailed t-test was performed ($P<0.05$) to assess statistical differences.

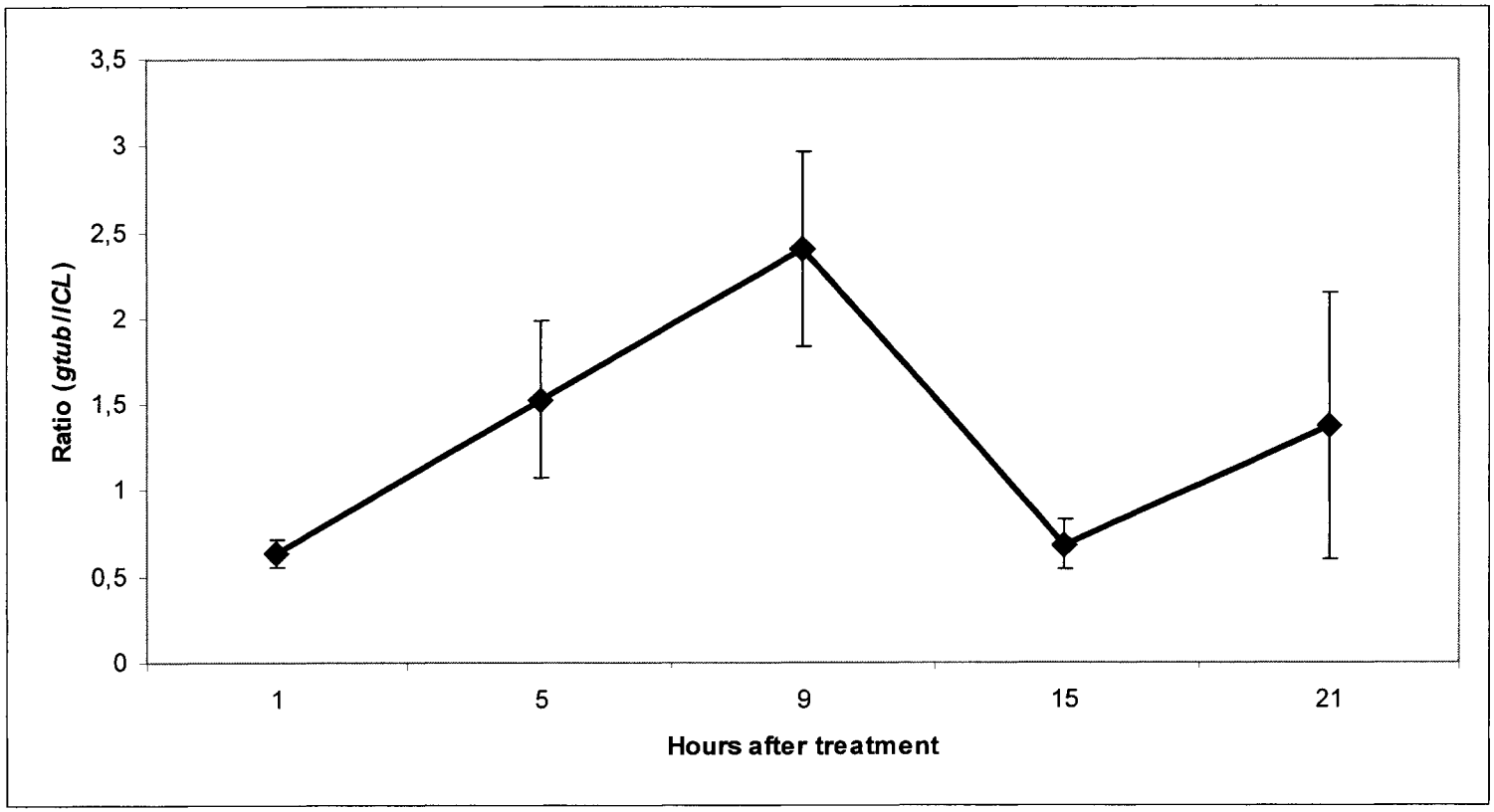


Figure 15. Soybean *ICL1* relative expression at different times after LCO treatment and normalized with *gtub* analyzed using the Liu transformation method. Each point represents the mean of three replicates ± standard error. Relative gene expression was measured in soybean embryos collected at different times after the addition of LCO or water treatment in experiment #1. A ratio of one means that the number of *ICL1* transcripts from treated soybean was equal to the number of *ICL1* transcripts from the non-treated soybean. A two-tailed t-test was performed ($P<0.05$) to assess statistical differences.

Within the same experiment, both the Pfaffl and Liu methods yielded similar results and this was true for the three independent experiments (ex. Figures 12 and 14). The method described by Liu was reputed to be more accurate because it calculates the amplification efficiency from the actual slope of the amplification plot (Liu and Saint, 2002), whereas the Pfaffl method calculated the amplification efficiency by using the cDNA standard simple linear regression curve (Pfaffl, 2001). The major problem facing the approach proposed by Liu is that amplification efficiency changes throughout the PCR, with efficiency declining in later cycles as amplification products compete for DNA polymerase binding (Kainz, 2000). We observed that the standard error increased slightly using the Pfaffl method compared to the Liu method in all of the relative gene expression studies conducted on *cdc2*, *WASI*, *ICL1* and *14-3-3* genes in experiments #1, #2 and #3. However, the Pfaffl method was more representative when the gene was down-regulated. The amplitude of a gene down-regulated with the Liu method was compressed between "0" and "1", phenomenon that did not exist with the Pfaffl method. For example, a gene down-regulated by 8 fold will have a value of -8 with Pfaffl method and a value of 0.125 with the Liu method. For this reason, the method used for analysis of gene expression on soybean was the mathematical transformation proposed by Pfaffl.

2.5 Analysis of gene expression in soybean using a combined data set for the three independent experiments

Figure 16 shows the ratio of *ICL1* gene expression relative to *RPL13* between LCO and water treatments for three different experiments conducted in soybean, and transformed using the Pfaffl method. A ratio of zero meant that the amount of *ICL1* transcripts from treated soybean was equal to the amount of *ICL1* transcripts from non-treated soybean. In other words, LCO treatment did not increase gene expression at that time point. As shown in Figure 16, a statistically significant increase in *ICL1* gene expression by the LCO treatment was found at the 5hrs time point. However, Experiments #2 and #3 did not show similar results. The effect of LCO on *ICL1* transcription did not have the same impact; the increase that was observed in experiment

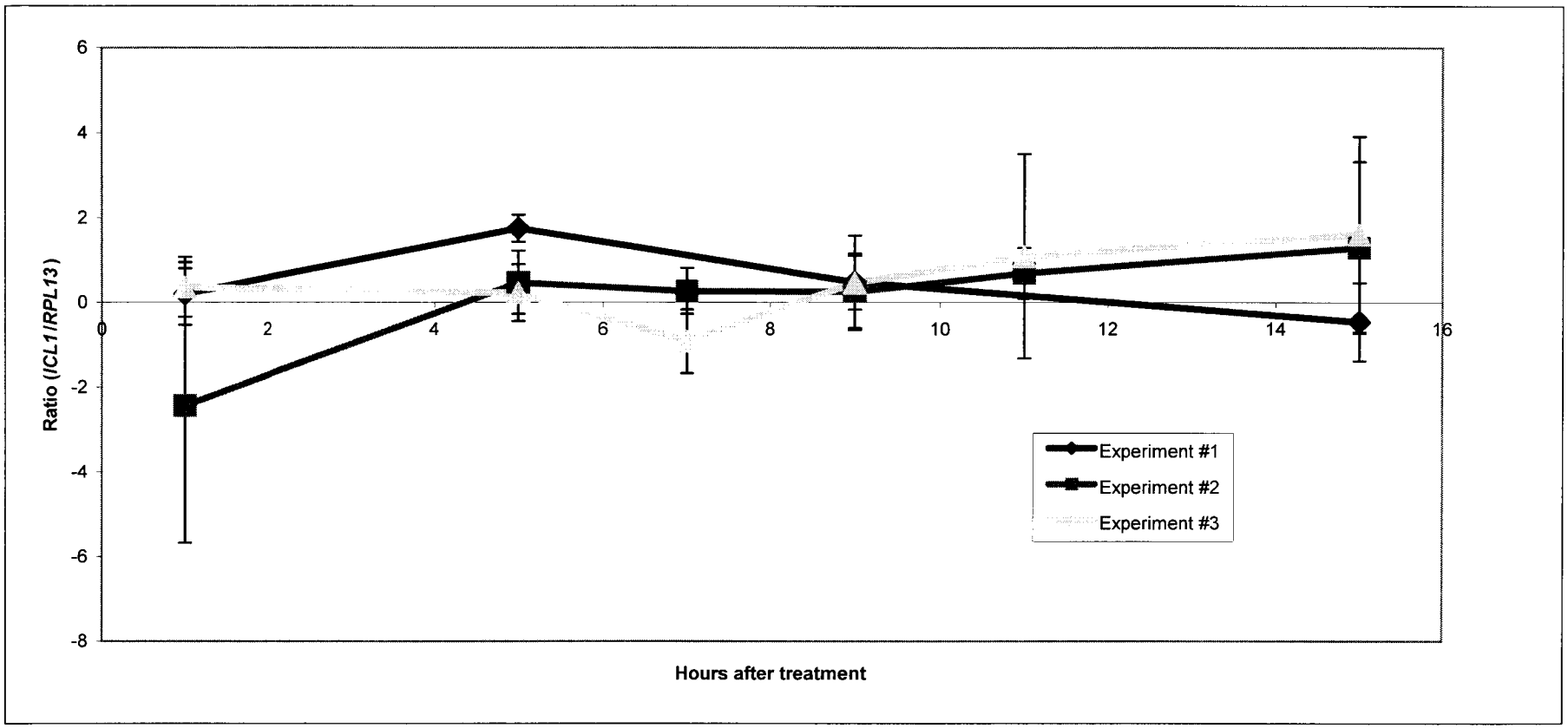


Figure 16. Soybean *ICL1* relative gene expression at different times after LCO treatment normalized with *RPL13* and analyzed using the Pfaffl transformation method from experiments #1, #2 and #3. Each point represents the mean of three replicates which contained ten embryos ± standard error. Gene expression was normalized using *RPL13* transcripts and the data analyzed with the Pfaffl method (Pfaffl, 2001). A two-tailed t-test was performed to assess statistical differences ($P<0.05$).

#1 was not shown in experiments #2 and #3. The same variation in results was observed for the three other genes *cdc2*, *WASI* and *14-3-3* also studied (data not shown). The three independent experiments did not show any trend in gene expression profile for the genes studied. Many factors can influence gene expression. The experimental conditions of the independent experiments were carefully controlled. The temperature was maintained at 24°C ± 1 in an incubator and the germination test was done in the dark. Given these observations, statistical analyses were performed to examine the possibility of combining gene expression data for the three experiments for each gene using a two-way randomized blocks analysis of variance (ANOVA) ($P<0.05$) and a least significant difference (LSD) method to determine significant difference among means. No statistically significant difference was observed between the results at specific time points between the three experiments, allowing us to combine the data already collected. Combining the three experiments increased the standard errors at some time points. The results were analyzed by using the Pfaffl model and normalized with the *RPL13* internal control. A two- tailed t-test was performed to test for the statistical significance changes in gene expression after LCO treatment.

The *ICL1* gene was chosen for these studies because mRNA expression of the *ICL1* gene was found to be induced following imbibition in *A. thaliana* (Eastmond and Graham, 2001; Rylott et al., 2001). Furthermore, it was proposed that isocitrate lyase plays an important role in controlling the rate of flux of carbon from acetate to sugar (Runquist and Kruger, 1999). The isocitrate lyase enzyme is part of the glyoxylate cycle which is involved in the conversion of lipid to sugar during seed germination (Beevers, 1980; Kornberg and Beevers, 1957). Soybean is known to have a high oil content (Eastmond and Graham, 2001). During germination, seed storage reserves are transformed to metabolites that are transported around the seedling and used to sustain growth and respiration (Bewley and Black, 1985). The glyoxylate cycle plays a role in soybean seed germination (Eastmond and Graham, 2001). Therefore, it was hypothesized that a stimulation of germination will need a faster conversion of stored oil found in cotyledons to sugar in order to support radicle emergence from the seed coat. This may increase gene expression of *ICL1* relative to the control. Figure 17 shows gene

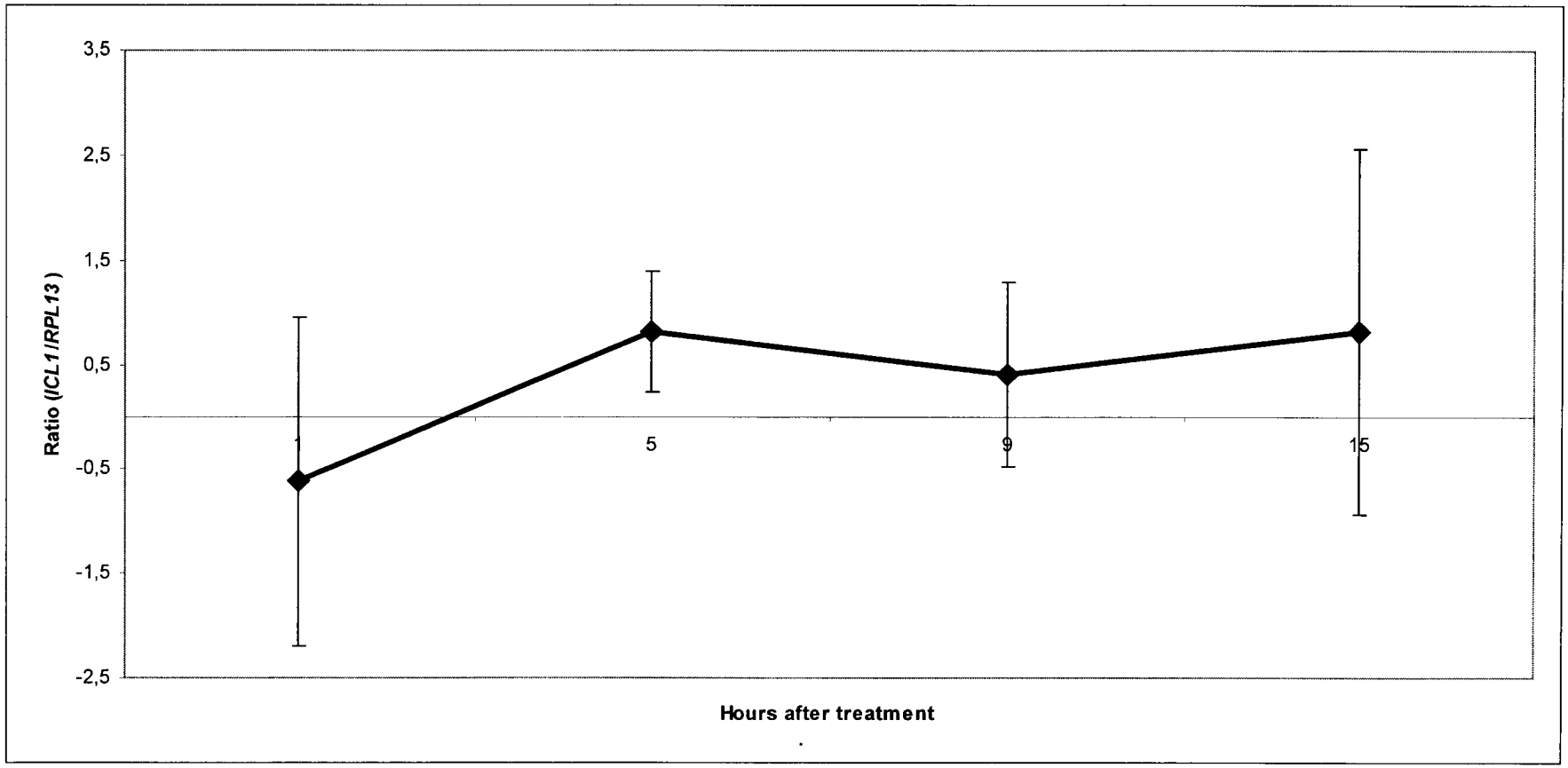


Figure 17. *ICL1* relative gene expression normalized with *RPL13* and calculated using the Pfaffl transformation method for the three experiments combined on *G. max* seed treated with 10^{-8} M LCO or water (control). Each point represents the mean of three independent experiments having three replicates each ± standard error. A two-tailed t-test was performed to assess statistical differences ($P<0.05$).

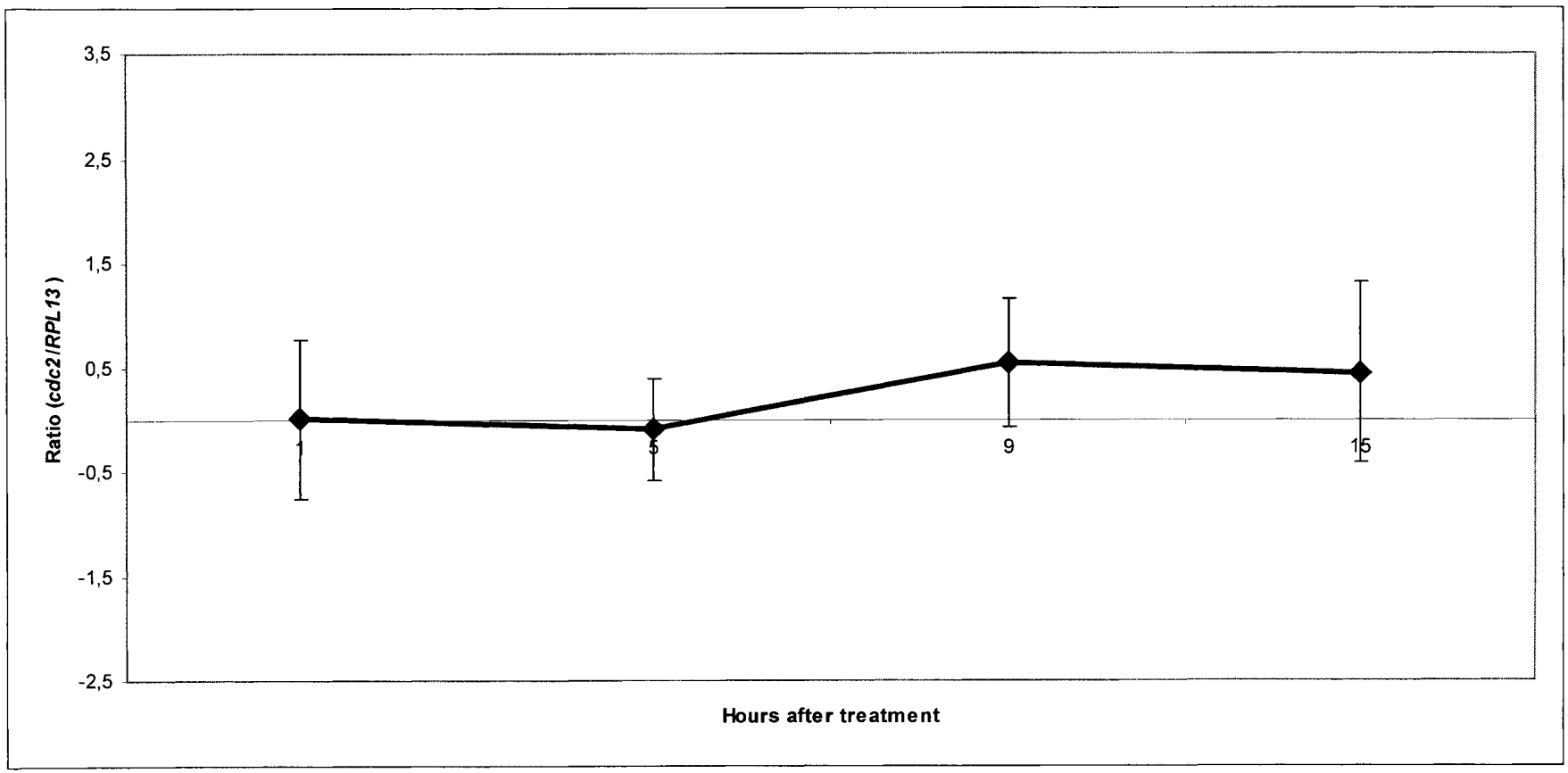


Figure 18. *cdc2* relative gene expression normalized with *RPL13* and calculated using the Pfaffl transformation method for the three experiments combined on *G. max* seed treated with 10^{-8} M LCO or water (control). Each point represents the mean of three independent experiments having three replicates each ± standard error. A two-tailed t-test was performed to assess statistical differences ($P<0.05$).

expression of *ICL1* relative to *RPL13* and transformed with the Pfaffl method for the three experiments combined. Statistical analysis showed that the ratio of gene expression following LCO treatment was not different from 0, meaning that LCO treatment did not have a statistically significant effect on *ICL1* expression at any collected time point.

We studied a gene that is part of the cell division cycle family because it was demonstrated that Nod factors stimulated cell division in the inner cortex during formation of the nodule primordium (Spaink et al., 1991; Truchet, 1991). It was also demonstrated that genes from this family were activated during germination in barley (Potokina et al., 2002). It was already known that the *cdc2* gene was induced in pea and alfalfa root cortical cells after addition of purified Nod factors (Yang et al., 1994). However, induction of the *cdc2* gene was not demonstrated during seed germination. It was hypothesized that the *cdc2* gene was also induced in the dividing cells of seed responding to the LCO induction. Figure 18 presents *cdc2* relative gene expression normalized with *RPL13* and calculated using the Pfaffl method for the three experiments combined. As shown, relative gene expression levels were stable for time points 1 and 5hrs after treatment, and no difference was found between water and LCO treatments using a two-tailed t-test statistical method ($P<0.05$). Statistical analysis showed that the ratio of gene expression following LCO treatment was not different from 0 for the 9 and 15hrs time points, meaning that LCO treatment did not have a statistically significant effect on *ICL1* expression at any collected time point.

The gene encoding the 14-3-3 protein was also selected because it was known to be induced during imbibition of germinating barley embryos (Testerink et al., 1999). Testerink and colleagues suggested a role for the 14-3-3 protein in signal transduction pathways during seed germination. H^+ pumping is likely to be implicated in these processes (Testerink et al., 1999). 14-3-3 is known to bind to H^+-ATPase (Fullone et al., 1998). It was proposed that the 14-3-3 protein may have a role in the regulation of H^+-ATPase in the scutellum during germination (Testerink et al., 1999). Figure 19 presents gene expression of the *14-3-3* gene relative to *RPL13* transcripts and analyzed using the Pfaffl method for the three experiments combined. The amount of transcripts was

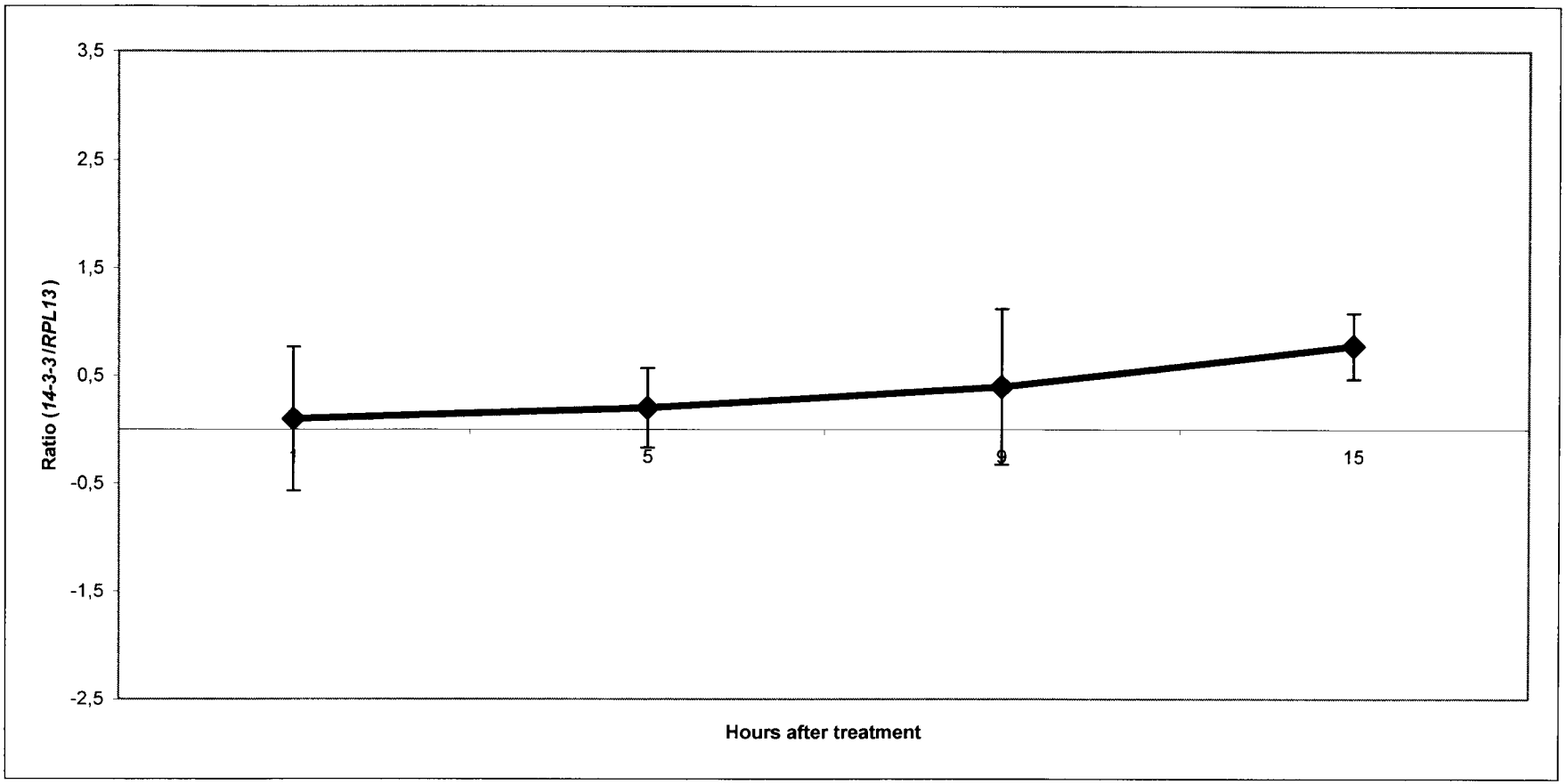


Figure 19. *14-3-3* relative gene expression normalized with *RPL13* and calculated using the Pfaffl transformation method for the three experiments combined on *G. max* seed treated with 10^{-8} M LCO or water (control). Each point represents the mean of three independent experiments having three replicates each ± standard error. A two-tailed t-test was performed to assess statistical differences ($P<0.05$).

increased for time points 1, 5 and 9hrs following the addition of LCO, and no difference was found between water and LCO treatments. However, a significant increase in LCO-treated samples was detected at 15hrs. The magnitude at which the variation of relative gene expression is considered biologically important is different between reports, but is commonly considered to be two-fold (DeRisi et al., 1997; Wildsmith and Elcock, 2001). In the case of *14-3-3* gene after 15hrs of LCO treatment, the increase in ratio was only about 0.78, not enough to be considered an important variation in gene expression induced by LCO treatment, even though the increase was statistically significant. The increase in *14-3-3* gene expression after LCO treatment coincided with radicle emergence from the seed coat at 15hrs. This increase was expected by studying the *14-3-3* profile obtained in the literature. It may be interesting to extend sample collection to have more data on *14-3-3* gene expression after 15hrs of exposure to LCO.

The *WASI* gene, that encodes an α-amylase inhibitor, was expected to show reduced expression over time (Potokina et al., 2002); this enzyme is involved in the regulation of endogenous α-amylase action (Henry et al., 1992; Henry et al., 1993). It is known that *α-amylase* expression is induced in the aleurone layer during cereal seed germination and plays a role in hydrolyzing the endosperm starch into metabolizable sugars which supply the energy for the growth of roots and shoots (Akazawa and Hara-Mishimura, 1985; Beck and Ziegler, 1989; Karrer et al., 1991). It was demonstrated that α-amylase activity increased after corn seeds were treated with LCO, triggering a more rapid breakdown of endosperm-stored starch reserves (Prithiviraj et al., 2003), giving us an indication of a pathway that may change after the addition of LCO on seeds. Figure 20 shows gene expression of *WASI* transcripts relative to *RPL13* transcripts calculated using the Pfaffl method for the three experiments combined. Statistical analysis showed that the ratio of gene expression following LCO treatment was not different from 0 for the 1, 5, 9 and 15hrs time points, meaning that LCO treatment did not have a statistically significant effect on *WASI* expression at any collected time point. A decrease in *WASI* gene expression was expected. Carbohydrates are not the major source of storage reserve in soybean (Eastmond and Graham, 2001). However, it was hypothesized that α-amylase

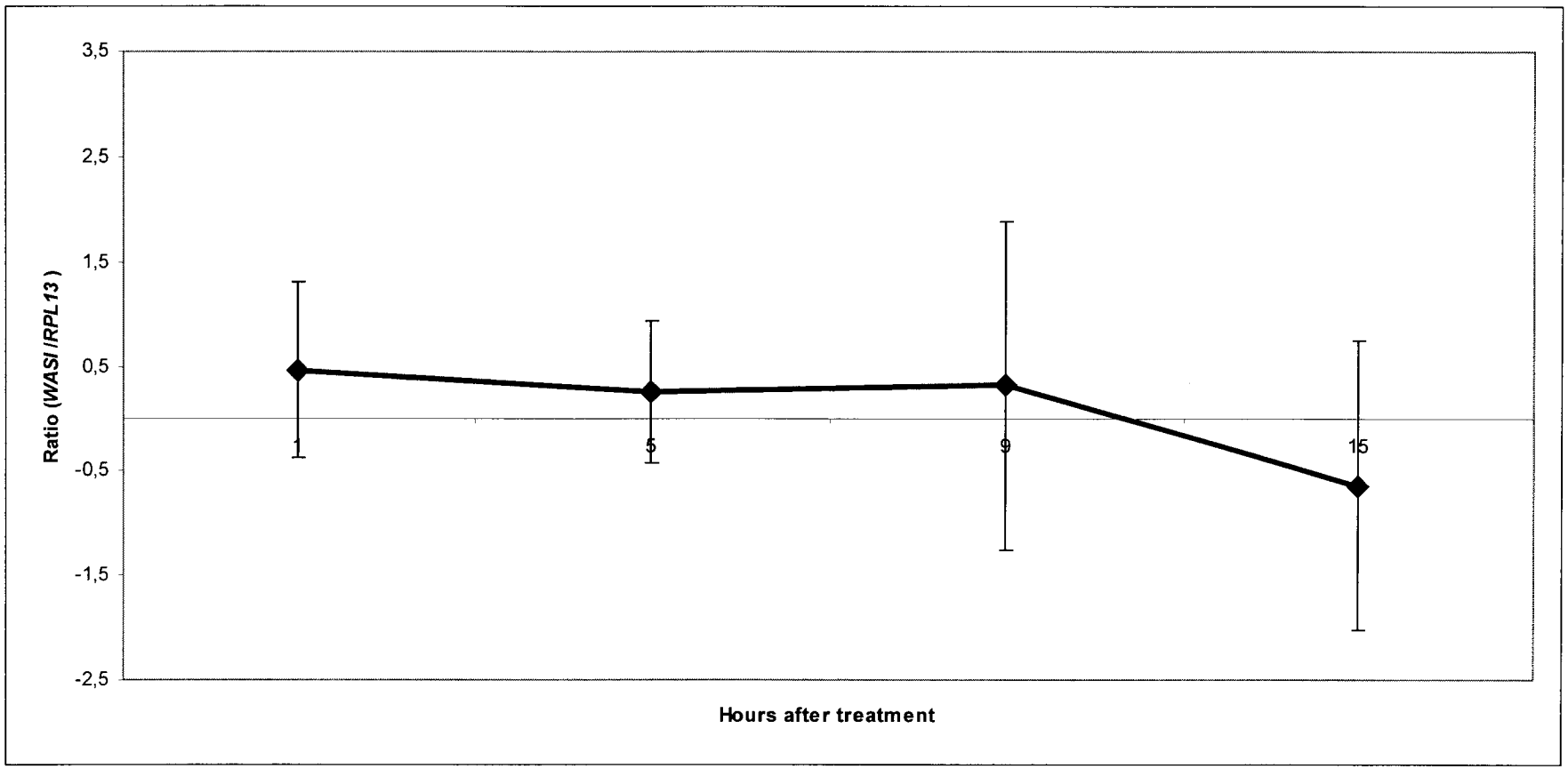


Figure 20. *WASI* relative gene expression normalized with *RPL13* and calculated using the Pfaffl transformation method for the three experiments combined on *G. max* seed treated with 10^{-8} M LCO or water (control). Each point represents the mean of three independent experiments having three replicates each ± standard error. A two-tailed t-test was performed to detect statistical differences ($P<0.05$).

will be required during soybean germination to hydrolyze the starch. Presence of α-amylase was reported in other legumes (Forsyth and Shewry, 2002).

2.6 Protein profiling of soybean after LCO treatment by SDS-PAGE

Since no statistically significant difference in gene expression for *cdc2*, *WASI*, *ICL1* and *14-3-3* genes was observed between the water and 10^{-8} M LCO treatments, an overview of global protein synthesis after LCO treatment was obtained. This part of the study was performed in order to investigate whether LCO has an impact on soybean protein profile by influencing for example the mRNA turnover, the translation or the posttranslational modifications. One-dimensional SDS-PAGE was used as a tool to look at the protein profile of soybean seeds after LCO application. Figure 21 shows an SDS-PAGE performed with proteins extracted from soybean seeds treated with water or 10^{-8} M of LCO. Two time points were tested in duplicate for each treatment. As clearly seen in Figure 21, no major protein change was observed between seeds treated or not with LCO. LCO did not appear to affect protein synthesis in the experimental conditions tried. 2-dimensional gel electrophoresis was also performed in order to check whether a major change in protein expression could be observed between water and LCO-treated seeds. No major change was observed between the two treatments (data not shown). The limitation of 2-D electrophoresis is the high variation in spot patterns between two gels, making the distinction of any true biological variation from experimental variation difficult (Alban et al., 2003).

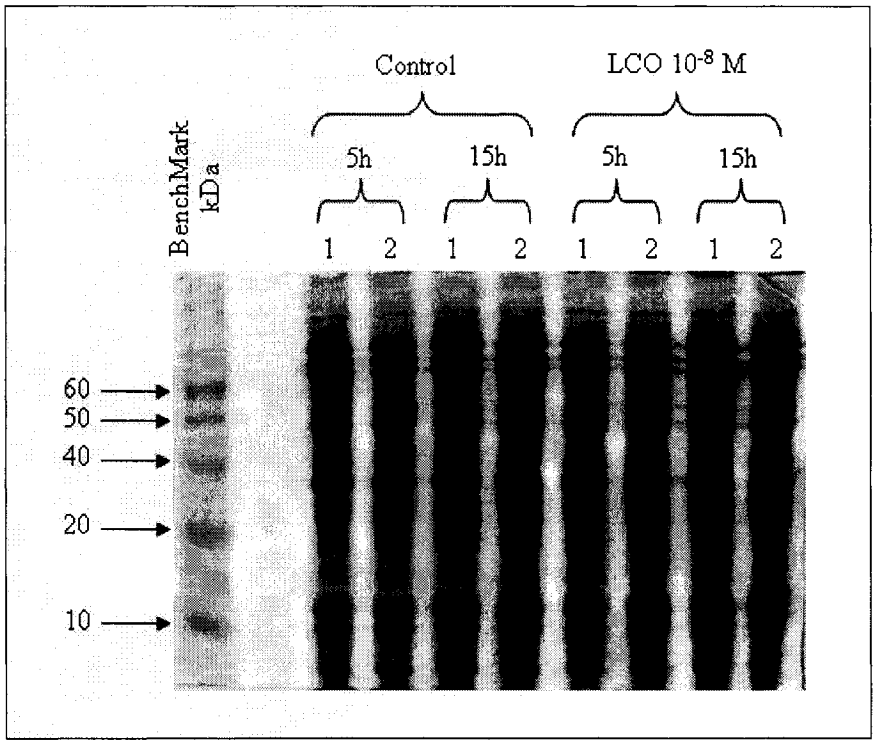


Figure 21. SDS-PAGE with proteins extracted from soybean seeds treated or not treated with LCO 10^{-8} M. Numbers above each well represent the replicates for 5 and 15hrs after addition of water or LCO treatment. 30 μg of BenchMark protein ladder were used.

CONCLUSION

LCO was found to produce different physiological responses on host and non-host plants such as promoting early growth of soybean and corn, stimulating seed germination and increasing biomass (Prithiviraj et al., 2003; Souleimanov et al., 2002). It has also been demonstrated that LCO enhances photosynthesis under greenhouse and field conditions (Donald L. Smith, personal communication; Khan et al., in press).

We first conducted experiments in tobacco to test for phenotypic changes following LCO treatment. No statistically significant differences were observed between the LCO and water-treated plants for photosynthetic rate, leaf area and dry weight. Acetonitrile was found by others to induce physiological changes in plants (Donald L. Smith, personal communication). The use of water only to dissolve LCO instead of a mix of acetonitrile and water could have affected the phenotypic response to LCO observed compared to the observations made in preceding studies. Since the purification of LCO using HPLC could have left contaminants, a mixture of LCOs in previous works could have led to a different phenotypic response in plants after LCO treatment compared to our experiments conducted with a different LCO product. It was also speculated that LCO may work by activating a general defense responses. These defense responses could also have been activated by insects during our experiments, as a large insect infestation was observed. Therefore, the presence of insects during the assays could have stimulated the defense response of plants before treatment application and then affected the plant response to LCO. Studies of effects of the LCO on plants were then conducted on soybean seed in order to assess gene expression profiles following LCO treatment.

We observed a stimulation of soybean seed germination after LCO addition, and therefore the addition of purified Nod factors modified the cellular response during the early stages of germination. Our hypothesis was that LCO application on a seed would modify gene expression correlated with a stimulation of germination by the treatment. The present study did not allow us to demonstrate a variation of gene expression related

to the enhancement of germination for the genes studied. None of the increases in gene expression was greater than two-fold. Furthermore, the time points that showed a significant variation in gene expression did not show the response we had hypothesized. Also, a significant variation was observed generally only for one time point.

The number of genes studied was small, therefore it is possible that LCOs act at the transcriptional level on other genes (Bauer et al., 1996; Fobert et al., 1996; Heidstra et al., 1997; Horvath et al., 1993; Minami et al., 1996). These previous studies were conducted with genes that were involved in the nodulation process, i.e. genes that were not usually expressed in plants, such as early nodulin genes (ex. ENOD40) and leghemoglobin gene. In our studies, genes were involved in normal germination process. A different gene expression profiling technique could have been used in order to have a more global profile of gene expression such as microarrays or cDNA AFLP (amplified fragment length polymorphism).

Specific changes in gene expression after LCO application for the genes studied could have been masked by the pooling of ten seeds per replicate. The expression profile of a specific cell type may be masked by the averaging of the expression of different cell types (Chelly et al., 1989). The number of seeds that responded to LCO treatment within a replicate was unknown.

An overview of global protein synthesis was also conducted by one-dimensional SDS-PAGE on soybean proteins extracted from seeds treated or not treated with LCO. No global protein change involved in the enhancement of germination was observed in the experimental conditions tried. It would be interesting to extend the protein profiling research using two-dimensional difference gel electrophoresis (Ettan^{TM} DIGE) (Alban et al., 2003).

Enhancement of germination following LCO application has been observed for a short period. The molecular events involved in these processes are still unknown. However, from this research, it seems that LCO does not modify gene expression profile

and protein profile compared to the control. In future experiments, it would be interesting to look at the post-translational modifications or enzyme activity.

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