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Molecular Signalling in *Lupinus albus-Rhizobium lupini* Symbiosis.

Hubert Gagnon

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy at

Concordia University

Montréal, Québec, Canada

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ABSTRACT

Molecular Signalling in *Lupinus albus-Rhizobium lupini* Symbiosis.

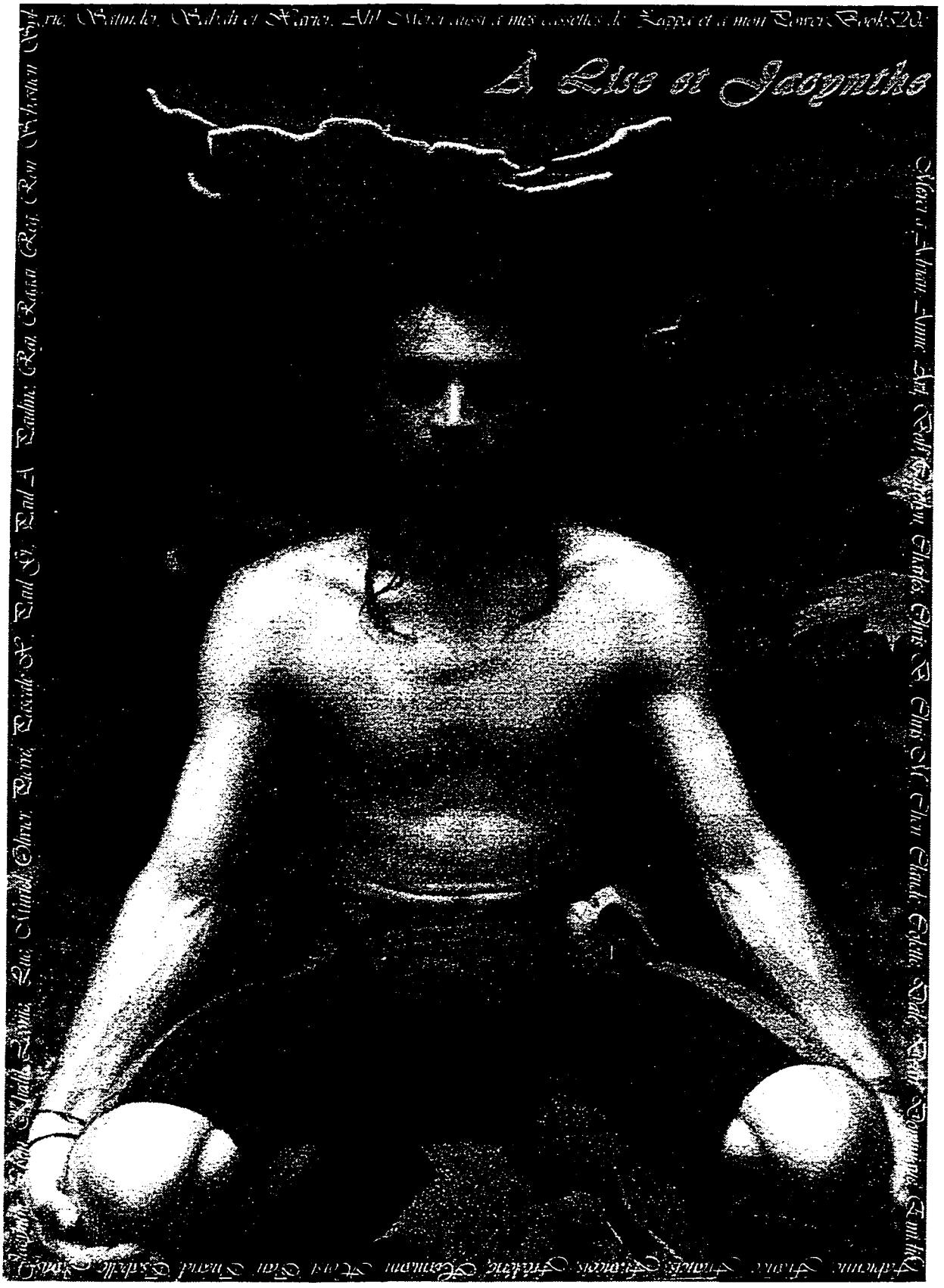
Hubert Gagnon, Ph.D.

Concordia University, 1997

The symbiotic interaction between *Rhizobium lupini* and *Lupinus albus* results in the formation of root nodules where nitrogen fixation takes place. In these associations, molecular signals such as flavonoids (or non-flavonoid type), *nod* gene-inducers, and bacterial lipochitooligosaccharides (LCOs) are known to act as modulators of species-specificity in the early stages of infection. Of the several known *Rhizobium*-legume symbioses, signalling in *Lupinus* remains to be determined.

Using a screening method based on the *in vitro* ability of the flavonoid inducer to adsorb onto the membranes of its symbiont, indicated that derrone, wighteone and lupiwighteone were adsorbed most onto *R. lupini* membranes. A second screening method, based on the ability of the inducer molecule to stimulate the growth rate of the symbiont, showed that 0.5 μM each of derrone, lupalbigenin, genistein monoprenyls, licoisoflavone A and lupinalbin A, resulted in a significant growth stimulation of *R. lupini* when cultivated in a minimal medium. A third strategy involved monitoring ß-galactosidase activity of *R. lupini* strains harboring *nodC::lacZ* fusions, in the presence of (a) authentic lupin isoflavones, (b) carbohydrate-like inducers, and (c) HPLC-fractionated lupin seed effusates and root exudates as putative *nod* gene inducers. The results indicated that both erythronic and tetronic acids (C4 sugar acids) led to low, but significant increases in ß-galactosidase activities, as compared with the controls. In addition, lupiwighteone, a monoprenylated isoflavone, seems to exert a synergistic effect with the carbohydrate-like inducers, as compared with other isoflavone treatments.

Incorporation studies of [^{14}C]LCO-precursors into induced *R. lupini* cultures, confirmed the inductive role of erythronic and tetronic acids, as well as their synergistic effect when combined with lupiwighteone or derrone. Enzymatic hydrolysis of the *R. lupini* LCOs with various enzymes substantiated their putative identities. Similar incorporation experiments were carried out on other *Rhizobium spp.* in order to assess the role of aldonic acids in promoting LCO biosynthesis. These results are discussed in relation to the impact of these unusual signal molecules on our knowledge of signalling in *Rhizobium*-legume symbiosis by flavonoids and LCOs.



Photograph by Francois Wells / Location: rainy hike in Val-David, Qc / Title: Hubert Gagnon docteur en biologie.

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Je voudrais avant tout remercier le Dr Ragai K. Ibrahim pour m'avoir permis de poursuivre ce projet de recherche dans son laboratoire. L'étendue de ses connaissances dans le domaine du métabolisme secondaire des plantes, son adaptabilité quant aux aspect du projet touchant à la microbiologie, son enthousiame et sa détermination en ont fait un superviseur hors pair. Je tiens a remercier le Dr Luc Varin pour son support intellectuel avisé et son expérience des études graduées. Je remercie le Dr Reginald Storms pour ses conseils judicieux.

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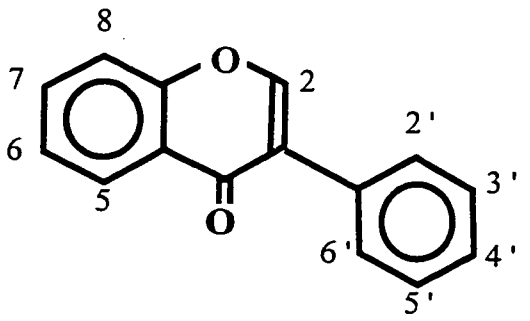
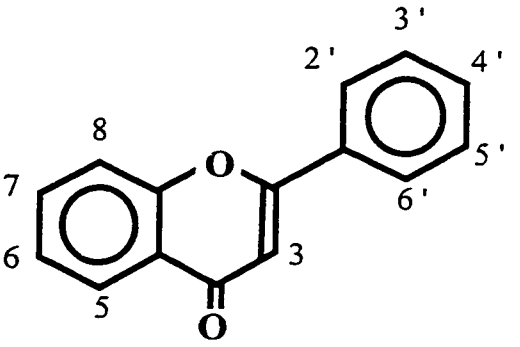


Isoflavone numbering system



Flavone numbering system

Compound name	Generic name
Apigenin	5,7,4'-Trihydroxyflavone
Biochanin A	4'-O-Methyl-5,7,-trihydroxyisoflavone
Daidzein	7,4'-Dihydroxyisoflavone
Derrone	7,8-Hydroxypyrano-5,4'-dihydroxyisoflavone
7,4'-Dihydroxyflavone	7,4'-dihydroxyflavone
Genistein	5,7,4'-Trihydroxyisoflavone
2'-Hydroxygenistein	5,7,2',4'-Tetrahydroxyisoflavone
2'-Hydroxylupalbigenin	6,3'-Diprenyl-5,7,2',4'-tetrahydroxyisoflavone
Isowighteone	3'-Prenyl-5,7,4'-trihydroxyisoflavone
Isoprunetin	5-O-Methyl-7,4'-trihydroxyisoflavone
Kievitone	8-Prenyl-5,7,2',4'-tetrahydroxyisoflavone
Licoisoflavone A	3'-Prenyl-5,7,2',4'-tetrahydroxyisoflavone
Lupalbigenin	6,3'-Diprenyl-5,7,4'-trihydroxyisoflavone
Lupinalbin A	5,7,4'-Trihydroxycoumaronochromone
Lupiwighteone	8-Prenyl-5,7,4'-trihydroxyisoflavone
Luteolin	5,7,3',4'-Tetrahydroxyflavone
Luteone	6-Prenyl-5,7,2',4'-tetrahydroxyisoflavone
Naringenin	5,7,4'-Trihydroxyflavanone
Prunetin	7-O-Methyl-5,4'-trihydroxyisoflavone
Quercetin	3,5,7,3',4'-Pentahydroxyflavone
Wighteone	6-prenyl-5,7,4'-Trihydroxyisoflavone

LIST OF ABBREVIATIONS

Abbreviation	Name
Ac	Acetyl
AmOH	Amyl alcohol
n-BuOH	*n*-Butanol
bv	biovar
B.	*Bradyrhizobium*
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
EtOH	Ethanol
EA	Erythronic acid
GalN	Galactosamine
GlcN	Glucosamine
HOAc	Acetic acid
HPLC	High pressure liquid chromatography
Iso-PrOH	*Iso*-propanol
leg.	*Leguminosarum*
L.	*Lupinus*
LCO	Lipochitooligosaccharide
ManN	Mannosamine
MeOH	Methanol
NaOAc	Sodium acetate
nod	Nodulation
OD	Optical density
PDA	Photodiode array
R.	*Rhizobium*
RP	Reverse phase
Rt	Retention time
SA	Succinic acid
TA	Tetronic acid
TLC	Thin layer chromatography
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume

A. INTRODUCTION

Symbiosis between legumes and *Rhizobium spp.* results in the formation of root nodules, which are structurally and biochemically specialized in nitrogen fixation (Long, 1989). These result in the high protein content of legumes; thus contributing to nitrogen availability in soils and are, therefore, of significant ecological and nutritional importance (Krishnan and Pueppke, 1992). These symbiotic interactions depend primarily on the ability of the host to form root nodules (Phillips, 1992; Stafford, 1997), and since these are mostly restricted to legumes, it would be of interest to extend the *Rhizobium* host-range to other important crop plants.

The molecular basis of these symbiotic interactions has become an intensive field of research during the last decade. One important finding of this research has been the recognition that some flavonoid compounds act as molecular signals, synthesized by the host plant and secreted in the rhizosphere, thereby activating bacterial infection (Table 1; Long, 1989; Phillips, 1992). These signals were reported to act as chemoattractants of *Rhizobium* (Caétano-Annolès *et al.* 1988), as well as inducers of bacterial nodulation (*nod*) genes (Peters *et al.* 1986). It should be noted, however, that other non-flavonoid type molecules such as betaines have also been shown to act as *nod* gene-inducers in *Rhizobium meliloti* (Figure 1A; Phillips *et al.* 1994). In addition, other flavonoid compounds were reported to act as chemorepellants of *Rhizobium* (Caétano-Annolès *et al.* 1988), and to inhibit the expression of *nod* genes (Peters and Long, 1988). Furthermore, the specific flavonoid-inducing signals are capable of adsorbing onto the bacterial membranes (Recourt *et al.* 1989; Hubac *et al.* 1993) and of acting as *in vitro* growth stimulators of the symbiont (Hartwig *et al.* 1991).

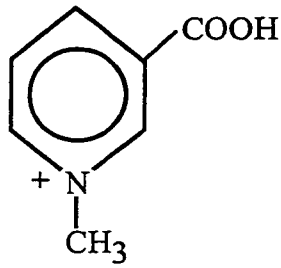
Flavonoids secreted in the rhizosphere by a compatible host activate the bacterial NodD protein, a transcriptional activator, resulting in the expression of all other bacterial nodulation genes (Fisher and Long, 1992). These genes code for enzymes involved in the biosynthesis or secretion of lipochitooligosaccharides (LCOs) which act as bacterial nodulation signals (e.g. *Nod* factors; Table 2; Figure 1C; Carlson *et al.* 1994). In addition,

Table 1. Species-specific symbiotic pairs and their molecular determinants

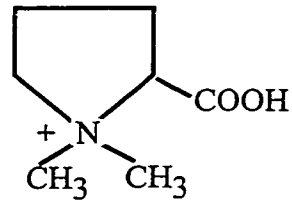
Host (latin name)	Host (common name)	Symbiont	Flavonoid inducer	Flavonoid inhibitor	Ref.
Lupinus albus	White lupin	*R. lupini*	ND	ND	--
Lupinus albus	White lupin	*B. lupini*	ND	ND	--
Lotus pedonculatus		*R. loti*	ND	ND	--
Medicago sativa	Alfalfa	*R. meliloti*	Luteolin	Apigenin	1, 2, 3
			Chrysoeriol	Naringenin	2, 3, 4
			4-4'-DiOH-2'-methyl chalcone		5
Trifolium repens	White clover	*R. l.* bv *trifolii*	Luteolin	Daidzein	6
			7,4'-DiOH-	Formononetin	6
			flavone;		6
			Hesperitin		7
Glycine max	Soybean	*R. fredii*	Daidzein	ND	8
Glycine max	Soybean	*B. japonicum*	Daidzein	ND	9
Phaseolus vulgaris	Common bean	*R.l.* bv *phaseoli* (renamed *tropici*)	Delphinidin	ND	10
			Petunidin		10
			Malvidin		10
			Naringenin		10
Pisum sativum	Pea	*R. l.* bv *viciae*	Apigenin	ND	8
			Eriodictyol		8
			Luteolin		8
Vicia villosa	Vetch	*R. l.* bv *viciae*	ND	ND	--

1, Mulligan and Long, 1985; 2, Peters *et al.* 1986; 3, Firmin *et al.* 1986; 4, Maxwell *et al.* 1989; 5, Hartwig *et al.* 1990; 6, Redmond *et al.* 1986; 7, Dénarié *et al.* 1992; 8, Sadowsky *et al.* 1988; 9, Kosslak *et al.* 1987; 10, Hungria *et al.* 1991. ND, not determined.

A. Structure of betaines

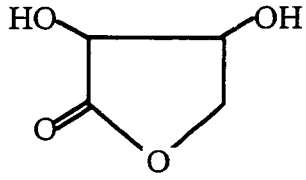


Trigonelline

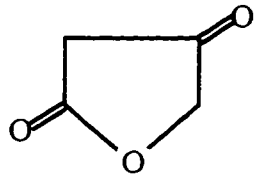


Stachydrine

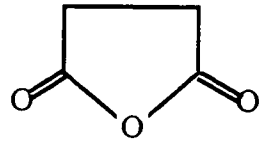
B. Structure of aldonic acids



Erythronic acid



Tetronic acid



Succinic acid
(lactonized)

C. Structure of the LCO (*Nod* factor) backbone
Refer to Table 2 for ***n*** and R1 to R4 residues.

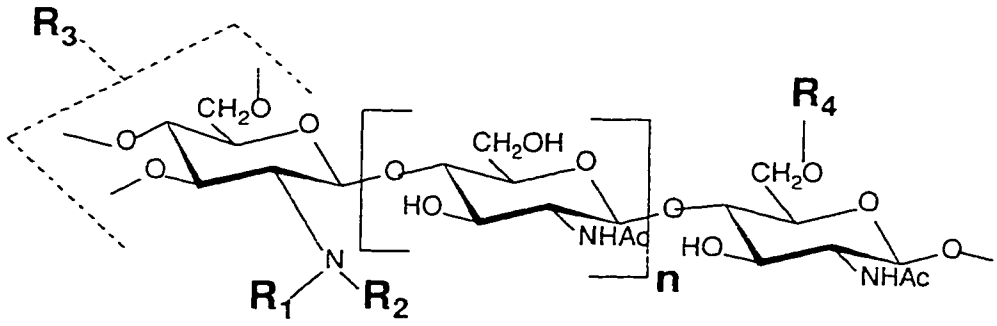


Figure 1. Molecular structure of known (A) and putative (B) *nod* gene inducers, and of nodulation factors (LCOs; C)

Table 2. Substituents reported for various rhizobial *Nod* factors

Rhizobium and *Bradyrhizobium* species	R1*	R2	R3	R4	*n* **	Ref.
R. meliloti	H	C16:2	Ac(*O*-6)	Sulfate	1,2,3	1
	H	C16:3	H	Sulfate	1,2,3	2, 3
R. l. bv *viceae*	H	C18:4	Ac(*O*-6)	H	2,3	4
	H	C18:1	Ac(*O*-6)	Ac(*O*-6)	2,3	
R. tropici	Me	C18:1	H	Sulfate	3	5
R. fredii	H	C18:1	H	MeFuc	1,2,3	6
	H	C18:1	H	Fuc	1,2,3	6
B. japonicum	H	C18:1	Ac(*O*-6)	MeFuc	3	7, 8
	H	C16:0	H	MeFuc	3	8
	H	C16:1	H	MeFuc	3	8
B. elkanei	H	C18:1	Ac(*O*-6)	MeFuc	2,3	8
	Me	C18:1	H	Fuc	2,3	8
	Me	C18:1	Cb	Fuc	2,3	8
R. loti	Me	C18:1	Cb	AcFuc	3	9
	Me	C18:0	Cb	AcFuc	3	9
R. lupini	ND	ND	ND	ND	ND	ND

*R1 to R4 correspond to the positions shown in Figure 1C.

***n*, number of glucosamine residues minus two

1, Lerouge *et al.* 1990; 2, Roche *et al.* 1991b; 3, Schultze *et al.* 1992; 4, Spaink *et al.* 1991; 5, Poupot *et al.* 1993; 6, Beckferte *et al.* 1993; 7, Sanjuan *et al.* 1992; 8, Carlson *et al.* 1993; 9, Lopez-Lara *et al.* 1995. Abbreviations: R, residue; C16:0, palmitic acid; C18:1, vaccenic acid, etc.; Ac, acetyl; Cb, carbamoyl; Fuc, fucosyl; Me, methyl; ; ND, not determined.

LCOs are known to contribute physically to the attachment to, and curling of root-hair cells of the host, thus acting as the modulators of species-specificity upon host infection (Carlson *et al.* 1994).

Therefore, both flavonoids (as well as some betaines) and LCOs are considered signal molecules that determine species-specificity in these symbioses (Fisher and Long, 1992; Phillips, 1992), and this specificity is achieved by specific substitutions of either the flavonoids or LCOs. In order to screen for unknown flavonoid inducers, induction of *nod* gene expression has been examined through the use of reporter genes, such as *lacZ*, inserted downstream of the *nodABC* operon (Zaat *et al.* 1989). However, flavonoid inducers can also be investigated using other approaches, such as their ability to adsorb onto *Rhizobium* membranes (Hubac *et al.* 1993), and the stimulatory effects they have on the *in vitro* growth rate of the symbiont (Hartwig *et al.* 1991). Furthermore, the biosynthesis and accumulation of LCOs can be used as a screening method to recognize the putative *nod* gene inducer(s) (Lortet *et al.* 1996).

Lupinus albus (white lupin; Figure 2A), a member of the Leguminosae, is capable of establishing a symbiosis with *Rhizobium lupini** and *Bradyrhizobium lupini* (Table 1; Figure 2B). The roots of white lupin have the ability to synthesize a variety of isoflavonoid aglycones and glucosides, as well as their mono- and diprenylated derivatives (Figure 3), and the phytochemistry of these isoflavonoids has been well studied (Harborne *et al.* 1976; Igham *et al.* 1983; Tahara *et al.* 1984; 1985; 1989; 1990; Shibuya *et al.* 1991; Gagnon *et al.* 1997). Most of these compounds were shown to be secreted into the rhizosphere of the intact roots (Gagnon *et al.* 1995b), as well as into the nutrient medium of *in vitro* cultured lupin cells (Gagnon *et al.* 1992b). In contrast with the majority of legumes, white lupin tissues constitutively synthesize and accumulate prenylated isoflavones (Harborne *et al.* 1976; Gagnon *et al.* 1992b; Gagnon *et al.* 1995b), and may thus be considered as pre-infectional compounds. In fact, some of these compounds have been reported to have a fungitoxic effect (Harborne *et al.* 1976), and were recently shown to retard the *in*

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* Listed as *R. loti* in the ATCC catalog (#35173; NZP2196 or SU343), but since it was isolated from *Lupinus* species, it is designated as *Rhizobium lupini* throughout the text.

A

B

Figure 2. Photographs of *Lupinus albus*(A) and of *R. lupini* cultivated in a Petri dish (B)

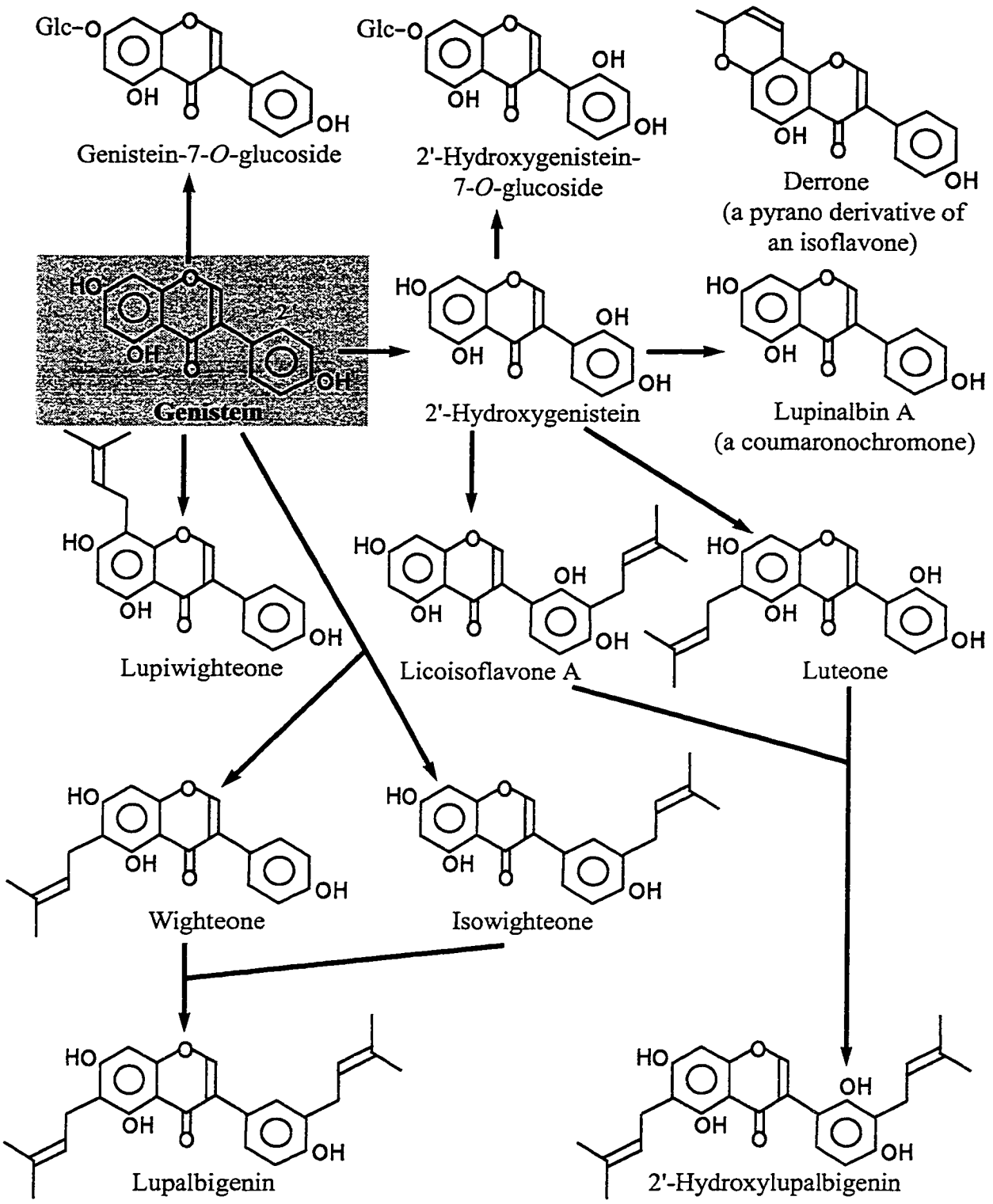


Figure 3. Chemical structures of lupin isoflavonoids

vitro growth rate of *R. lupini* cultured in a complex medium (Gagnon, 1993). In addition, white lupin roots contain glycoproteins whose glycosidic moiety consists of 30% erythronic acid (Figure 2B; Dr. R. Ibrahim, unpublished results).

In contrast with several other *Rhizobium*-legume associations, both the flavonoid and LCO signals which confer specificity of lupin symbiosis with *R. lupini* are yet to be identified (Lopez-Lara *et al.* 1995; Stafford, 1997). Therefore, we propose as the major goal of this work to characterize these two types of molecules. While signalling features similar to other systems should apply to the *Lupinus-Rhizobium* symbiotic interaction, this bacterium has to cope with the host's endogenous pool of pre-infectional isoflavonoids. Therefore, white lupin and *R. lupini* are considered very attractive candidates for the study of molecular signalling of their symbiosis, with the ultimate aim of possibly increasing the diversity of known symbiotic signals (flavonoid inducers and *Nod* factors).

B. AIM OF WORK

The main objective of my doctoral studies were to determine which of the lupin metabolites, isoflavonoid-type or other, is responsible for *nod* gene induction and which LCO (*Nod* factor) is synthesized as a result of *nod* gene expression in *Rhizobium lupini*. In other words, my goal is to characterize the two signal molecules conferring species-specificity in the *R. lupini-L. albus* symbiosis.

Two indirect approaches were taken as a preleminary step in this investigation. The first approach takes advantage of the known ability of flavonoid inducer molecules to bind onto the membranes of the symbiont in other *Rhizobium sp*. This was carried out in order to study the differential *in vitro* adsorption of putative isoflavonoid inducers of *R. lupini*. The other indirect approach to the study of *nod* gene inducibility by isoflavonoids in *R. lupini* is based on the known stimulatory effect of flavonoid inducers on the *in vitro* growth rate of the symbiont when cultivated in minimal media.

A direct approach to study *nod* gene-inducibility in *R. lupini* by lupin isoflavonoids is to transform *R. lupini* cells with a cosmid harboring a *nodABC::lacZ* fusion (e.g. pRmM57; Mulligan and Long, 1985). The transformed strains will then be treated with putative isoflavone-inducers, and assayed for ß-galactosidase activity as a measure of *lacZ* expression and, thereby of *nod* gene induction. An alternative screening approach is to demonstrate the *de novo* synthesis of *Nod* factors in *R. lupini,* after induction with the lupin *nod* gene inducer, by *in vitro* incorporation of ^{14}C-labeled LCO precursors. In addition, the identity of the radiolabeled *Nod* metabolites can be confirmed by their sensitivity to known LCO-degrading enzymes. Furthermore, similar *in vitro* assays with putative *nod* gene inducers of the symbiont will also be carried out in order to monitor *Nod* factor accumulation and, after purification, its biological activity on lupin root hairs.

Finally, if the newly discovered inducer is not an isoflavone of *L. albus*, then the presence of another putative inducer in lupin tissues, and its significance in the signalling process will be confirmed.

C. REVIEW OF LITERATURE

C.1. *RHIZOBIUM*-LEGUME INTERACTIONS

C.1.1. Classification and Genetics of *Rhizobium*

Rhizobium species are Gram-negative motile rods that live freely in soils (Vincent, 1970), and their relative abundance increases in the vicinity of the compatible host (Taylor and Beringer, 1981). The majority of *Rhizobium spp.* were, until recently, classified into two separate genera, *Bradyrhizobium* and *Rhizobium*; a classification based on their characteristic slow or fast growth rates, respectively. Species classification is related to the host(s) each strain is able to nodulate in a species-specific manner as shown in Table 1 (Long, 1989). The genes controlling the early and late functions of symbiosis in *Rhizobium spp.* were found to be located on a megaplasmid (pSym), in the absence of which these bacteria lose the ability to nodulate their host (Rosenberg *et al.* 1981; Marvell *et al.* 1987). *Rhizobium* megaplasmids range in length from 200 Kb in *R. leguminosarum* to 1500 Kb in *R. meliloti* (Long, 1989).

In light of the new advances in *Rhizobium* genetics, a reclassification system has been proposed based on the location of nodulation genes (Scott *et al.* 1996), as well as the homologies among glutamine synthase II gene sequences (Taboada *et al.* 1996). This led to the inclusion of a new genus, *Mesorhizobium* (Martinez-Romero and Caballero-Mellado, 1996; E. Martinez-Romero, Chair of the International Committee for Taxonomy of *Rhizobium* and *Agrobacterium*, Personal Communication). In fact, the nodulation genes of *Rhizobium* and *Bradhyrhizobium* are located either on the symbiotic plasmid (Marvell *et al.* 1987) or on the genome (Banfalvi *et al.* 1981; Masterson *et al.* 1985), respectively; whereas, in the *Mesorhizobium,* they are located on both of these DNA molecules (Parry *et al.* 1994; Scott *et al.* 1996). In addition, members of *Mesorhizobium* (i.e. *R. leg.* bv *phaseoli*, *R. etli*, *R. loti* and *R. lupini*) secrete significant amounts of exopolysaccharides (Bailey *et al.* 1971; Grant and Scott, 1991; M. Hungria, Personal Communication); a

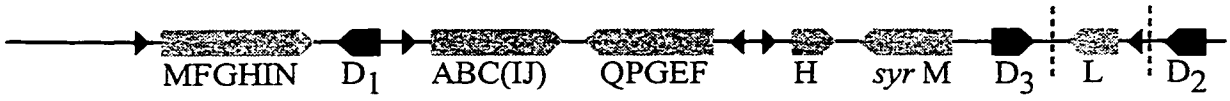
characteristic feature not found in most other *Rhizobium spp.* Furthermore, amino acid sequence comparisons of *Rhizobium nodC*s, and of legume-host leghemoglobins corroborate such reclassification; since although the evolutionary distance between *R. loti* and *R. leg.* bv *phaseoli* is small, they are less related to other *Rhizobium spp.* (Ueda *et al.* 1995). Nevertheless, these bacterial species will be referred to as *Rhizobium* throughout the text. It should be noted, however, that the growth of both *R. loti* and *R. lupini* result in a characteristic acidification of their environment, and are referred to as acid-producing strains (Crow *et al.* 1981; Jarvis *et al.* 1982).

C.1.2. The Symbiotic Genes and the Infection Process

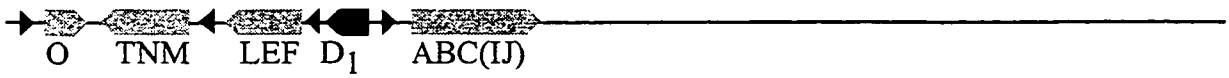
In 1980, the availability of a new cosmid vector (pLAFR1; Ditta *et al.* 1980) that is compatible with Gram-negative bacteria, made it possible to construct a gene bank from *R. meliloti* pSym (Friedman *et al.* 1982; Djordjevick *et al.* 1983). This enabled researchers to map and sequence several nodulation genes in *R. meliloti* (Rosenberg *et al.* 1981), as well as in other *Rhizobium spp.* (Figure 4; Hirsh, 1992). The characterization of several nodulation mutants was correlated with the different stages of *Rhizobium* infection (Debellé *et al.* 1986). Some classes of mutations were found to be required for the early steps of infection (e.g. root hair deformation, *Had*; root hair curling, *Hac*; cortical cell division, *CrD*; and nodule initiation, *NoI*), and are referred to as 'early nodulation genes' (Long, 1989; Hirsch, 1992).

Among the early nodulation genes, the *nod* genes (Mulligan and Long, 1985) confer host specificity and are required for *Hac*, *Had, CrD*, and *NoI* phenotypes. While *nod* genes do induce a host response, other early genes including those which produce exo- and lipopolysaccharides (*exo* and *lps* genes; Gray and Rolfe, 1990) are considered to act as 'avoidance determinants' of the plant defense system (Nap and Bisseling, 1990), and are involved in host cell penetration and infection thread formation (*Inf*; Gray and Rolfe, 1990). Other genes encoding enzymes involved in nodule function, such as nitrogen

Nod-genes of *Rhizobium meliloti*



Nod-genes of *Rhizobium leguminarosum* bv. *viciae*



Nod-genes of *Bradyrhizobium japonicum*

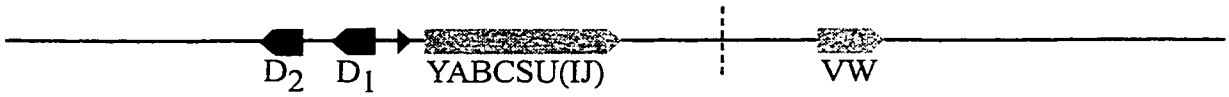


Figure 4. Physical map of *Rhizobium nod* genes

With the exception of *syr* (symbiotic regulator), all letters refer to *nod*-genes. The black triangles refer to *nod*-boxes, while the dashed vertical lines designate large interruptions in the map. The genes are not drawn to scale. From Hirsch, 1992.

fixation enzymes (*ntr* and *fix*), are not involved in host specificity (Long, 1989).

C.1.3. Structure, Inducibility and Regulation of *Nod* Genes

The *nod* genes of *Rhizobium* are arranged in 3-6 operons, each of which is preceded by a *Nod* box (Figure 4; Hirsch, 1992). The *Nod* box is a 47-bp long, strictly conserved region located in the extended promoter region of each *nod* operon in several *Rhizobium spp.* (Horvath *et al.* 1987; Rostas *et al.* 1986), and acts as a common promotor sequence for *Rhizobium nod* operons (Horvath *et al.* 1987). However, the constitutively expressed, or self regulated, *nodD* gene lies upstream from the *Nod* box of the *nod ABC(IJ)* operon, and is transcribed in opposite directions (Mulligan and Long, 1985; Rossen *et al.* 1985; Burn *et al.* 1987). In addition, multiple *nodD* genes are found in *R. meliloti* (Ausubel *et al.* 1987; Honma *et al.* 1990), *R. leguminosarum* bv *phaseoli* (Davis and Johnson, 1990), *B. japonicum, R. fredii* (Appelbaum *et al.* 1988) and *R. loti* (Scott *et al.* 1996), whereas other species such as *Rhizobium leg.* bv *viciae* only have a single *nodD* copy (Spaink *et al.* 1987). The first *nod* operon to be characterized was found to contain the *nodABC(IJ)* genes (Banfalvi *et al.* 1981). These genes were called the 'common *nod* genes', since their sequence is highly conserved in all *Rhizobium spp.* (Friedman *et al.* 1982), and because they functionally complement similar genes in other *Rhizobium spp.* (Long *et al.* 1982). The other *nod* genes, constituting other operons, are referred to as the 'host-specific genes', since they are not present in all *Rhizobium spp.*, although their expression depends on the transcriptional activation by the NodD protein (Long, 1989).

The first case of *nod* gene induction was reported by Mulligan and Long (1985). Their pioneering work was carried out with *R. meliloti*. In order to monitor the expression of *nodD1* and *nodABC(IJ)*, they used *R. meliloti* strains transformed with translational *lacZ* fusions (and Tn5 insertions; Djordjevic *et al.* 1985) and an assay for ß-galactosidase activity in the presence (or absence) of alfalfa exudates. Their assays revealed that *nodD1*, as well as alfalfa exudates, were required for *nodC* expression, since the pRmM67

construct (*nodABC(IJ)::LacZ*; *nodD::Tn5*) did not yield any ß-galactosidase activity as compared with constructs which expressed functional nodD1 (i.e. pRmM57; Mulligan and Long, 1985). These results suggested a role for *nodD* in the regulation of the expression of other *nod* operons. The experimental system elaborated in the latter study proved useful for the investigation of the chemical nature of the inducer(s) responsible for *nodABC* expression in *R. meliloti* was isolated from alfalfa root exudates (Peters *et al.* 1986). This was accomplished by fractionating alfalfa exudates on HPLC, and assaying the fractions for *nodABC::lacZ* inducing activity in a transformed strain of *R. meliloti* (pRmM57). The most active fractions were further purified and the inducer was identified as luteolin (3',4',5,7-tetrahydroxyflavone, Table 1), based on ultraviolet absorption spectra, proton nuclear magnetic resonance, and mass spectral analyses (Peters *et al.* 1986). In addition, synthetic luteolin also exhibited inducing activity, and its spectral characteristics were found similar to those of the natural alfalfa inducer. The natural *nod* gene inducer was found to be active in the range of 0.01 μM to 10 μM concentrations (Peters *et al.* 1986).

Other investigations using similar translational *lacZ* fusions were conducted with exudates from various leguminous hosts and their compatible *Rhizobium spp.*, and led to the discovery of the *nod* gene inducers in other symbiotic systems (Zaat *et al.* 1989). The various examples shown in Table 1 demonstrate the wide diversity of flavonoids which can act as inducers of *nod* genes, and includes flavones, isoflavones, flavonols, flavanones, dihydroflavonols and anthocyanidins. In addition, several inducers may be exuded naturally from a host root, each of which may have a different inducing potential. This phenomenon has been well illustrated by testing three *Rhizobium spp.* harboring *nodABC::lacZ* fusions in the presence of all compounds exuded from *Vicia sativa* (Zaat *et al.* 1989). The results showed that different flavonoid compounds resulted in a wide range of *nod* gene-induction levels, as determined by ß-galactosidase assays. In addition, these studies revealed a strict requirement for a 7-hydroxylation (flavone numbering system) and,

to a lesser extent, a 4'-OH group for *nod* gene induction. The requirement for a 7-OH was also reported in an earlier study of *R. leg.* bv *trifolii* (Redmond *et al.* 1986). Whereas most flavonoid inducers are hydroxylated aglycones, their corresponding glycoconjugates, also found in the root exudates, were reported to have a much lower inducing activity (Hartwig and Phillips, 1991). However, there are some reported cases where methylated derivatives of chalcones (2'-*O*-methylisoliquiritigenin) and flavones (chrysoeriol), as well as methylated flavanones (hesperitin) were found to act as *nod* gene inducers in *R. meliloti* (Maxwell *et al.* 1989; Hartwig *et al.* 1990) and *R. leg.* bv *viciae* (Dénarié *et al.* 1992), respectively. Therefore, lipophilic flavonoids can also act as *nod* gene inducers.

In contrast with most *nod* gene-inducers which belong to the flavonoids, stachydrine and trigonelline, both of which are betaine derivatives (i.e. heterocyclic N-compounds that consist of 4 or 5 carbons, respectively; Figure 2A), were also shown to act as *nod* gene inducers in *R. meliloti* (Phillips *et al.* 1992). Furthermore, this research group showed that, after monitoring ß-galactosidase activity with *nodC::lacZ* fusions in various *nodD* deletion mutants of *R. meliloti*, the *nodD2* was required for *nod* gene induction by betaines (Phillips *et al.* 1992), whereas the *nodD1* and the *nodD3* is known to respond to flavonoids (Honma *et al.* 1990; Mulligan and Long, 1989). However, in order to achieve induction levels comparable to those of luteolin in *R. meliloti*, a thousand-fold higher concentration of either of these betaines was required (Phillips *et al.* 1994). Furthermore, these newly discovered inducers were shown to be secreted by alfalfa seedlings at a 10^3-fold higher concentration, as compared to that of luteolin (Phillips *et al.* 1992). Betaines have been reported to occur in intact *Medicago sativa*, as well as its cell suspension cultures (Sethi and Carew, 1974; Phillips *et al.* 1992).

C.1.4. *Nod* Genes: Regulation of Expression

The *nodD* amino terminal sequences are highly conserved among different *Rhizobium spp.*, and they show high homologies to the *LysR* family which is known to encode DNA binding domains (Burn *et al.* 1987). It was later shown that gel retardation of promotor regions of *nod* genes, in the presence of NodD protein, indeed takes place (Hong *et al.* 1987). Another line of evidence demonstrated that binding of the *nodD* product to the *nodA* promotor does not depend on the presence of the inducer (Fisher *et al.* 1988). These features suggest that the NodD protein is a DNA-binding protein which acts as a transcriptional regulator of *nod* gene expression. In addition, the construction of chimeric *nodD* carboxy terminal from different *Rhizobium spp.* alters the flavonoid specificity of the resulting proteins (Horvath *et al.* 1987; Spaink *et al.* 1989). These results confirm that the carboxy terminal is involved in the specificity of activation by flavonoids, and allowed to design new cosmid constructs that encode Flavonoid-Independent Transcriptional Activator *nodD*s (i.e. FITA *nodD*; Spaink *et al.* 1989).

C.1.5. Other Characteristics of *Nod* Gene Inducers

Other reports have demonstrated that naringenin (the flavanone inducer of *R. leg.* bv *viciae*, naturally exuded from its host; Table 1) accumulates on the cytoplasmic membrane of *R. leg.* bv *viciae* without apparent metabolic conversion (Recourt *et al.* 1989). Since inducible *nod* genes are not required for naringenin accumulation on the cell surface (Recourt *et al.* 1989), and that the NodD protein is localized on the cytoplasmic membrane (even in the absence of the host exudate; Schlaman *et al.* 1989) it is assumed, therefore, that naringenin binds to this protein (VanBrussel *et al.* 1990). Another similar report showed that luteolin is adsorbed at a much higher rate onto membranes of the alfalfa symbiont, *R. meliloti*, as compared with other flavonoids that are not *nod* gene inducers in *R. meliloti* (Hubac *et al.* 1993). The latter authors hypothesized that luteolin adsorption

was taking place with a certain degree of species-specificity (Hubac *et al.* 1993), that can be used in the screening for inducer molecules in other *Rhizobium spp.*

C.1.6. *Nod* Gene Products and *Nod* Factors (LCOs)

When all *nod* genes are expressed in *Rhizobium spp.* the resulting proteins are involved either in the biosynthesis, or the secretion, of precursor molecules that constitute a bacterial nodulation signal termed the *Nod* factor (Roche *et al.* 1991b). These molecules consist of ß-1,4-linked oligomers of 3 to 5 *N*-acetylglucosamine residues with an amide-linked fatty acyl moiety on the non-reducing terminal residue (Table 2; Figure 1C; Carlson *et al.* 1994). In view of their chemical structure, their resemblance to chitins, and their biological activity, these molecules are referred to as lipochitooligosaccharides (LCOs), also known as nodulation factors (*Nod* factors; Spaink, 1994).

The first reported bacterial *Nod* factor was isolated from luteolin-induced *R. meliloti* cells, and termed NodRm1 (Table 2; Lerouge *et al.* 1990). This factor was extracted with *n*-BuOH, resuspended in water and partitioned against ethyl acetate. It was further purified by gel permeation and ion-exchange chromatography, followed by HPLC. Its presence was monitored during the purification process by its ability to induce root hair curling on alfalfa. Analysis by mass spectrometry, nuclear magnetic resonance, ^{35}S-labelling, and chemical modification studies revealed a 6-*O*-sulfated *N*-(C16:1)acyl-tri-*N*-ß-1,4-D-acetylglucosamine tetrasaccharide (Roche *et al.* 1991b). The purified NodRm1 factor was active at nanomolar concentrations in root hair deformation of alfalfa, but not on vetch, a heterologous host (Lerouge *et al.* 1990; Truchet *et al.* 1991; Schultze *et al.* 1992).

C.1.7. Methods for Isolation of *Nod* Factors

Nod factors have been isolated from many other *Rhizobium spp.* (Figure 1C, Table 2), and a clear link has been established between the presence of *nod* gene inducers and the biosynthesis and accumulation of LCO by the symbiont (Carlson *et al.* 1994; Spaink,

1995). Protocols have been developed to assess LCO biosynthesis (Spaink *et al.* 1992; Price and Carlson, 1995; Lopez-Lara *et al.* 1995). These involved the use of radiolabeled LCO-precursors such as [^{14}C]glucosamine and [^{14}C]acetate, although the former precursor allows for a higher specific activity of the LCOs produced, as compared with the latter (Price and Carlson, 1995). Although some *Nod* factors are secreted by the bacteria, most remain embedded in the membranes (Orgambide *et al.* 1995). Nonetheless, most reports focused on the extracellular fraction for its ease of extraction (Carlson *et al.* 1994; Dénarié and Debellé, 1996). *Nod* factors were initially extracted from the supernatants of *exo* mutant cultures (i.e. deficient in exopolysaccharide production; Lerouge *et al.* 1990; Roche *et al.* 1991b), with *n*-BuOH as solvent (Promé *et al.* 1992; Poupot *et al.* 1993; Cardenas *et al.* 1995; to mention a few). In contrast with other chromatographic media, which were originally used to purify and characterize these metabolites (Roche *et al.* 1991b), HPLC on C18-RP columns is now routinely used as an efficient chromatographic step for the large scale purification of LCOs (Poupot *et al.* 1993; Cardenas *et al.* 1995; Folch-Mallol *et al.* 1996). In addition, the fact that LCOs absorb best at 206 nm (Schultze *et al.* 1992) allowed the use of acetonitrile as the HPLC solvent of choice, since it has a low UV cut-off (Poupot *et al.* 1993; Cardenas *et al.* 1996). Furthermore, the advent of photodiode array (PDA) detection technology in HPLC analysis, has enabled researchers to readily identify LCOs on the basis of their UV spectra. It should be noted that, upon HPLC elution, LCO peaks appear as multi-apex (Lopez-Lara *et al.* 1995; Cardenas *et al.* 1996). This chromatographic behavior has been attributed to the fact that LCOs may elute as a complex with micelles (i.e. remnants of the rhizobial membrane; Price and Carlson, 1995).

More recent techniques for the analysis of LCOs from *Rhizobium* culture supernatants involve the use of C18-silica Sep-Pack™ for their pre-purification, and fractionation by RP-C18 TLC, followed by autoradiography (Spaink *et al.* 1992; Learemans *et al.* 1996). An advantage of this technique is that it allows a higher LCO yield (Price and Carlson, 1995). Silica plates (Price and Carlson, 1995), as well as RP silica (i.e. derivatized with C18;

Spaink *et al.* 1992) have been used with different solvent systems in labeling studies. The use of RP Si-TLC plates combined with 50% aqueous acetonitrile as developing solvent give the best resolution, since the latter meets the requirements for LCO polarities on such a TLC support (Spaink *et al.* 1992). A combination of these techniques is now being used to screen for *nod* gene inducers, as well as to compare the LCO patterns produced by different *Rhizobium* isolates (Lortet *et al.* 1996).

Finally, it should be noted that environmental factors, such as low pH, temperature and high nitrogen availability, which are known to reduce nodulation in the field, were also found to affect the *in vitro* production of *Nod* metabolites (Richardson *et al.* 1988; Dusha *et al.* 1989; McKay and Djordjevic, 1993; Mendosa *et al.* 1996).

C.1.8. How Signal Molecules from *Rhizobium* Determine Species-Specificity

Using immunocytochemical techniques, the NodC protein was localized in the inner bacterial membrane (Barny and Downie, 1993), and was found to function as a uridine diphosphoacetylglucosaminyltransferase that is involved in oligoacetylglucosamine synthesis (Debellé *et al.* 1992; Geremia *et al.* 1994), and determines chitooligosaccharide chain length (Dénarié *et al.* 1996). The NodB protein deacetylates the non-reducing end of the oligoacetylglucosamine (John *et al.* 1993; Spaink *et al.* 1994), whereas the NodA protein was found to function as an *N*-acyltransferase (Debellé *et al.* 1987; Rohrig *et al.* 1994). In addition, based on sequence homologies of the *nodE* and *nodF,* it is believed that their encoded proteins function as acyl carriers and in the reduction or condensation of acyl groups with the oligoglucosamine, respectively (Spaink *et al.* 1991). Sequence homologies of the *nodIJ* suggest the involvement of their proteins in the transport of *Nod* factors across cytoplasmic membranes (McKay and Djordjevick, 1993; Carlson *et al.* 1994). Therefore, common *Rhizobium nod* genes encode enzymes involved in the biosynthesis of LCO backbones, whereas other *nod* genes that are specific to each

Rhizobium sp. will encode specific LCO-substitution enzymes (Carlson *et al.* 1994; Spaink, 1995; Dénarié *et al.* 1996).

The following example well illustrates how the host-range specificity can be altered when host-specific *nod* genes are mutated, and results in substituent alterations of LCOs. *R. meliloti* NodP and NodQ proteins are involved in adenosine 5'-phosphosulfate (APS) synthesis, as was demonstrated both *in vitro* and *in vivo* (Schwedock and Long, 1989; 1990). In addition, *nodH* is homologous to, and codes for, a sulfotransferase involved in the species-specific sulfation of NodRm1 (Faucher *et al.* 1989; Roche *et al.* 1991a). Another *Nod* factor (NodRm2) was isolated from *R. meliloti* mutants, at either the *nodH* or *nodQ* loci, which lacked the sulfate group (Lerouge *et al.* 1990). NodRm2 does not cause root hair curling of alfalfa, but causes root hair curling of vetch, when applied at concentrations of 10^{-8} to 10^{-9} M (Roche *et al.* 1991b). Therefore, specific modifications of the rhizobia-produced LCO appear to determine host specificity and, in most cases, these modifications were shown to originate from the expression of host-specific *nod* genes (Carlson *et al.* 1994).

Other structural variations influencing the biological activity and specificity of *Nod* factors (LCOs) are the *O*-acetylation of the non-reducing terminal residue (Bloemberg *et al.* 1994), *O*-arabinosylation (Mergaert *et al.* 1997) and *O*-fucosylation (Sanjuan *et al.* 1992; Stacey *et al.* 1994) of the reducing terminal residues, as well as the methylation and carbamoylation of the acylated amino groups (Poupot *et al.* 1993; Jabbourit *et al.* 1995; Lopez-Lara *et al.* 1995). Therefore, host-specific *nod* genes contribute to the changes in the structural features of *Nod* factors.

Variations in the fatty acid side chain length (i.e. C18 or C16), as well as the presence or absence of carbon to carbon double bonds, have also been reported by many investigators (Truchet *et al.* 1991; Carlson *et al.* 1994). Thus *cis*-vaccenyl-, palmityl-, palmitoleyl- and stearyl-LCO precursors have been reported to reflect the lipid composition of cell membranes (Dénarié *et al.* 1996), and it seems reasonable to assume that they may

not contribute to host specificity. In contrast with these common lipids, highly unsaturated LCO side chains have also been reported to exist (e.g. C20:4; Spaink *et al.* 1991), and it has been postulated that acyl groups may also act as determinants of host-specificity (Spaink *et al.* 1991; Spaink, 1992; Spaink *et al.* 1995). However, examples involving the C20 polyunsaturated side chains were later shown, after thorough spectral re-analysis to be the sodium adducts of common lipids (i.e. C18, C16; Philip-Hollingsworth *et al.* 1995), a controversy that is still being debated (Van Der Drift *et al.* 1996).

Similar *Nod* factors have been isolated from *R. loti* and *R. leg.* bv *phaseoli* (Lopez-Lara *et al.* 1995; Cardenas *et al.* 1995; respectively) and their molecular structure was reported to be 6-*O*-acetylfucose- 2-*O*-carbamoyl *N*-(C18:1)acyl-*N*-methyl-tetra-*N*-ß-1,4-D-acetylglucos- amine pentasaccharide. In addition, the host-specific *nod* genes that encode enzymes involved in specific substitutions of these LCOs have been cloned, sequenced and characterized. The *nodZ* encodes a methylfucosyltransferase in *B. japonicum,* or an acetylfucosyltransferase *in R. loti* (Sanjuan *et al.* 1992; Quinto *et al.* 1997), the *nodS* encodes an *S*-adenosyl-L-methionine-dependent methyltransferase in *R. sp.* NGR234 (Jabbourit *et al.* 1995), and *nodU* encodes a 6-*O*-carbamoyltransferase (Jabbourit *et al.* 1995).

Other compounds, such as delphinidin (an anthocyanidin), as well as naringenin and eriodyctiol (flavanones) are reported to act as *nod* gene inducers of *R. phaseoli* (Hungria *et al.* 1991), whereas the inducers of *R. loti* remain to be determined (Lopez-Lara *et al*, 1995; Stafford, 1997). In fact, several commercially available flavonoids*, as well as the betaine trigonelline, have been tested as putative *nod* gene inducers in *R. loti*, although without conducting any LCO biosynthesis (Lopez-Lara *et al.* 1995). Nevertheless, the production of LCOs by *R. loti* was achieved using a strain transformed with a FITA cosmid, although

* "Naringenin, luteolin, eriodyctiol, hesperetin, apigenin, 7-8-dihydroxyflavone, genistein, daidzein, 7-hydroxyflavone, umbelliferrone, dihydrorobinetin, dihydroquercetin, dihydrofisetin, kaempferid, pinocembrin, morin, scutellarein, kaempferol, prunetin, chrysin, 5-hydroxyflavone, 6-hydroxyflavanone, 3'-*O*-methyldihydroquercetin, malvidin, fisetin, geraldol, quercetin, and isosakuretin."

no inducer has yet been found. It has been hypothesized, therefore, that the *nod* gene inducer (i.e. the host signal) may be solely responsible for species-specificity (Long, 1996), since the *Nod* factors produced by these different *Rhizobium spp.* have similar chemical structures (Poupot *et al.* 1993; Lopez-Lara *et al.* 1995). This contrasts with what has been observed for other *Rhizobium spp.* (e.g. *R. meliloti* and *R. leg.* bv *viciae*) where the known inducers are similar, but different *Nod* factors are produced by the two different species (i.e. sulfated or not, Long, 1996). It appears, therefore, that the strict species-specificity in *Rhizobium*-legume interactions is modulated in some instances, by the structural specificity of plant factors whereas in others, it is modulated by the structural specificity of the *Nod* factor.

C.2. PUTATIVE INDUCER MOLECULES FROM *LUPINUS ALBUS*

C.2.1. The Isoflavonoids of *Lupinus albus*

The phytochemistry of *Lupinus* isoflavonoids has been extensively studied by Dr. S. Tahara's group at Hokkaido University, which resulted in the elucidation of more than 30 structural types of isoflavonoids, many of which were generously supplied to us as reference compounds for our investigations (Figure 3). Genistein, a 5,7,4'-trihydroxyisoflavone present in many leguminous species, was shown to be the precursor of all other 5-hydroxyisoflavonoids (Schröeder *et al.* 1979; Tahara *et al.* 1984; Hagmann and Grisebach, 1984). 2'-Hydroxylation of genistein yields 2'-hydroxygenistein (Ingham, 1972), and further cyclization of this hydroxyl group with C-2 of the isoflavone ring yields lupinalbin A, a simple coumaronochromone also present in *L. albus* (Tahara *et al.* 1985). Prenylated isoflavonoids were also isolated from *Lupinus spp.* as the 3'-prenyl (isowighteone), 6-prenyl (wighteone) and 8-prenyl (lupiwighteone) derivatives of genistein (Fukui *et al.* 1973; Tahara *et al.* 1984). Similarily, the 3'-prenyl (licoisoflavone A) and 6-prenyl (luteone) derivatives of 2'-hydroxygenistein were also isolated from *L. albus.*

However, the 8-prenyl derivative of 2'-hydroxygenistein (2,3-dehydrokievitone) is absent in white lupin (Tahara *et al.* 1984). Pyran derivatives of monoprenylated isoflavones are also found in white lupin, especially that of lupiwighteone (i.e. derrone; Figure 3), where the 8-prenyl group is cyclicized with the 7-hydroxyl group (Tahara *et al.* 1984; 1989). In addition, the 3',6-diprenyl derivatives of both genistein (lupalbigenin) and 2'-hydroxygenistein (2'-hydroxylupalbigenin), as well as the 7-*O*-glucosides of both aglycones were isolated from *L. albus* (Ingham *et al.* 1983; Tahara *et al.* 1989; Shibuya *et al.* 1991).

C.2.2. The Involvement of *L. albus* Isoflavonoids in Nodulation

The effect of several *L. albus* isoflavonoids on the growth of *R. lupini* in complex media has previously been investigated, and resulted in statistically significant growth inhibitions with a variety of prenylated isoflavones tested (ca. wighteone, licoisoflavone A, lupalbigenin, luteone and 2'-hydroxylupalbigenin), whereas none of the aglycones tested showed any significant effect (Gagnon, 1993). These results indicated that both prenylation and 2'-hydroxylation of isoflavones have synergistic effect on the inhibition of bacterial growth rates. In fact, growth inhibition increases with isoflavone prenylation at positions 6,3'-di > 6 > 3', whereas 2'-hydroxylation (2'-hydroxylupalbigenin > lupalbigenin) confers additional growth inhibition. Therefore, lupin isoflavonoids seem to affect the growth rate of the lupin symbiont, *R. lupini,* in both a substituent- and position-specific manner. The fact that isoflavone prenylation does not affect the growth rate of *R. meliloti*, whereas it inhibits that of *R. lupini*, illustrates the specificity of the effect these lipophilic compounds have on the compatible symbiont (Gagnon, 1993).

Soybean nodules have been shown to accumulate glyceollin I, a diprenylated pterocarpan (an isoflavonoid derivative; Karr *et al.* 1993), and the latter compound was found to be bacteriostatic towards *Bradyrhizobium japonicum* (Parniske *et al.* 1991). Our recent study of the biosynthesis and accumulation of prenylated isoflavones in *L. albus* root

nodules, indicates that 2'-hydroxylupalbigenin is the predominant compound found in the nodule tissues (Gagnon *et al.* 1995a). However, when grown under sterile conditions (Gagnon *et al.* 1995b), or when infected with a *R. lupini* cell-suspension (Gagnon and Ibrahim, 1997), the roots of *L. albus* seedlings secreted respectively 10- and 40-fold more genistein monoprenyls (e.g. wighteone) than those of 2'-hydroxygenistein (e.g. luteone). Considering the fact that inhibition of bacterial growth increases with isoflavone prenylation and 2'-hydroxylation (i.e. 2'-hydroxylupalbigenin > luteone > wighteone; Gagnon, 1993), a relationship seems to exist between the different symbiotic events and the production of specific isoflavonoid conjugates which, in turn, differ in their inhibitory effect on the *in vitro* growth of the symbiont.

Taken together, these findings strongly suggest the involvement of white lupin isoflavonoids in the symbiotic process, and demonstrate that the structural diversity of these compounds may enable this plant to cope with *Rhizobium* infection (during early or late symbiosis) at various levels. The fact that these phenomena take place in a substituent-specific manner justifies the studies on the inducibility of *nod* genes in *R. lupini*.

C.2.3. Other Lupin Metabolites as Putative *Nod* Gene Inducers of *Rhizobium lupini*

In view of the fact that *L. albus* isoflavonoids act as weak *nod* gene inducer in *R. lupini* (results of this study), other compounds of natural occurrence in lupin should also be tested. The fact that leguminous roots are known to contain lectins and glycoproteins (Sharon and Lis, 1990; Peumans and VanDamme, 1995), prompted co-workers in Dr. Ibrahim's laboratory to carry out a carbohydrate analysis of the acid hydrolysates of the lectin-like glycoprotein fractions from lupin roots. After trimethylsilane derivatization and gas chromatography-mass spectral analysis (GC-MS) conducted at the Complex Carbohydrate Research Center (Dr. R.W. Carlson, Athens, GA), the hydrolysates were found to contain an uncommon aldonic acid, erythronic acid, which amounted to 30% of the total glycosyl residue (Unpublished results; Figure 2B). In fact, this compound is an

unusual constituent of the glycosidic moiety of plant glycoproteins (Dr. R.W. Carlson, personal communication). In addition, tetronic acid, a closely related structural analog, is reported to occur in marine sponges and to possess antibiotic activity (Figure 2B; Lumsdon *et al.* 1992; Davis and Capon, 1994). It seems reasonable, therefore, to test these compounds as putative *nod* gene inducers in *R. lupini*, since aldonic acids and stachydrine share some structural similarities (Figure 2A, B). The lactonized form of succinate (Figure 2B), a well known aldonic acid analog, is a common primary metabolite. Succinylated exopolysaccharides reported to occur in *R. meliloti* (Glucksmann *et al.* 1993) exhibit specificity in host-symbiont interactions (Reuber and Walker, 1993). Therefore, there are good reasons to believe that these aldonic acids may play a role in signalling during host-symbiont recognition.

D. MATERIALS AND METHODS

D.1. GENERAL MATERIALS AND METHODS

D.1.1. Special Chemicals, Radiochemicals and Materials.

Lupin isoflavonoids were a generous gift from Dr. Satoshi Tahara, Dept. of Applied Bioscience, Hokkaido University, Japan. These compounds (Figure 1) have previously been isolated and identified by various spectroscopic methods (Fukui *et al.* 1973; Harborne *et al.* 1976; Ingham *et al.* 1983; Tahara *et al.* 1984; 1985; 1989; 1990; Shibuya *et al.* 1991). Apigenin, daidzein, 7,4'-dihydroxyflavone, luteolin, and naringenin (Table 1) were purchased from Spectrum Chemicals (Gardena, CA) or Roth (Karlsruhe, Germany). Tetronic, succinic and erythronic acids were purchased from Sigma-Aldrich (St-Louis, MO). Standard flavonoids and aldonic acids were solubilized in 80% aq. MeOH or water, respectively, then filter-sterilized by centrifugation in microspin filters™ (CanadaWide Scientific, Ottawa, ON) prior to use in bioassays with rhizobia.

[1-^{14}C]Sodium acetate (Spec. Act. 57.2 mCi·mmol^{-1}), and D-[1-^{14}C]glucosamine hydrochloride, [1-^{14}C]galactosamine hydrochloride, [1-^{14}C]mannosamine hydrochloride, [1-^{14}C]acetylglucosamine hydrochloride and [1-^{14}C]acetylgalactosamine hydrochloride (Spec. Act. 55 mCi·mmol^{-1}) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Reverse phase, high performance thin layer chromatography plates (HPTLC; C18 derivatized silica, 100% silanized, 250μM thick, 10cm x 10cm) were purchased from Sigma-Aldrich (St-Louis, MO). HPLC-grade MeOH, AcN, HOAc, and *n*-BuOH were used wherever mentioned, and purchased from Fisher Scientific (Mississauga, ON). Proteinase K and lyzozyme were purchased from Boehringer Mannheim (Laval, QC) and the other enzymes, from Sigma-Aldrich (St-Louis, MO).

D.1.2. Plant Material

Seeds of white lupin, *Lupinus albus* L. *cv.* Kievskij were generously supplied by Dr. Satoshi Tahara, and *cv.* Primovsky were purchased from Pelletier (Montréal, QC). All *L. albus* seedlings were grown under controlled, sterile conditions for the production of seed effusates and root exudates (Gagnon, 1993).

D.1.3. HPLC Analysis of Isoflavonoids, Aldonic Acids and LCOs

HPLC analyses were carried out on silica C18 columns, either semi-preparative Lichrospher™ (Merck, Darmstadt, Germany) or analytical Nova-Pack™ (Waters Millipore, Gilford, MA). Data aquisition based on photodiode array detection from 200 to 400 nm, and data processing were acheived using the Millennium™ workstation (Waters Millipore, Gilford, MA), which is supported by a Microsoft Windows™ 3.1 environment (Mississauga, ON). All HPLC elutions (isocratic or gradient) were carried out using aq. MeOH for isoflavone and aldonic acid analyses, or aq. AcN for LCO analyses. The UV absorbance of flavonoids and aldonic acids were monitored and calibrated at 280 nm and 220 nm, respectively, whereas LCOs were monitored at 206 nm. For quantification of compounds, a range (0.5 to 40 μL) of standard solutions of isoflavones (in 80% aq. MeOH) and of aldonic acids (in H_2O) were chromatographed, their absorbance monitored; and the eluted peaks exhibited linear signal responses (i.e. $r^2 > 0.995$) in the range of 0.5 to 40 nmoles (for flavonoids) or mmoles (for aldonic acids) from which extinction coefficients were deduced at 280 nm and 220 nm, respectively (Table 2).

D.2. RHIZOBIA: GROWTH AND TRANSFORMATION

D.2.1. Bacterial Strains and Cosmids

The wild-type strains utilized in this study are listed in Table 3. The 29.3 Kb-long pRmM57 cosmid (*nodC::lacZ* fusion; $Spec^{res}$, Tet^{res}) used to study *nod* gene inducibility

Table 3. Wild type strains utilized in this study

Bacteria	Source*		
	ATCC #	NZP #	Other #
Agrobacterium rhizogenes	-	-	LBA 9402
Bradyrhizobium japonicum	10324	-	NCIB11477
R. leguminosarum bv *phaseoli*	14482	-	-
R. leguminosarum bv *trifolii*	14480	-	-
R. leguminosarum bv *viciae*	10004	-	NCIB11478
Rhizobium loti	-	2235	CC812a
Rhizobium lupini	35173	2196	SU343
Rhizobium meliloti	9930	-	-

*Abbreviations: ATCC; American Type Culture Collection; NZP, New Zealand Patent.

was generously supplied by Dr. S. Long (Stanford University, CA) in an *E. coli* background (Mulligan and Long, 1985). The Flavonoid-Independent Transcriptional Activator *nodD* (FITA *nodD*) harboring cosmid p2112 ($Spec^{res}$) was used as positive control in the LCO-incorporation studies, and was obtained from Dr. H.P. Spaink (Leiden University, The Netherlands). *Rhizobium meliloti* 1021 harboring pRmM57 transformant was kindly supplied by Dr. D.A. Phillips (University of California, Davis).

D.2.2. Complex and Defined Growth Media for *Rhizobium lupini*

Yeast-mannitol liquid broth consisted of 0.4 $g \cdot L^{-1}$ yeast extract, 10 $g \cdot L^{-1}$ mannitol, 0.05% KH_2PO_4 (pH 6.8), 0.02% $MgSO_4 \cdot 7H_2O$ and 0.01% NaCl (w/v)(Vincent, 1970), and was used as a complex growth medium. In this medium, the growth of *R. lupini* was optimal at 28°C, with a doubling time of ca. 8.0 h.

Three defined minimal media were used, which contained various C and N sources. The MA medium, based on mannitol (1%, w/v) and NH_4Cl (0.01%, w/v); the GS medium, based on monosodium glutamate (3 mM) and monosodium succinate (6 mM); and the GM medium, based on monosodium glutamate (3 mM) and mannitol (1%, w/v). They are referred to as the MA, GS and GM media, respectively, throughout the text. Each of the latter media was supplemented with a formulation derived from Hartwig *et al.* (1991), and consisted of the following macronutrients: 2 mM KH_2PO4/Na_2HPO_4 (pH 6.8), 5 mM $MgSO_4 \cdot 7H_2O$, 5 mM $CaCl_2$, 10 ppm $FeCl_3$, and micronutrients (per liter): 1.0 mg H_3BO_3, 100 µg $ZnSO_4 \cdot 7H_2O$, 50 µg $CuSO_4 \cdot 5H_2O$, 50 µg $MnCl_2 \cdot 4H_2O$, 10 µg $Na_2Mo_4 \cdot 2H_2O$, 119 µg $NiCl_2 \cdot 6H_2O$, and supplemented with 1 $mg \cdot L^{-1}$ each of biotin, thiamine and pantothenate. A non-physiological buffer, MOPS (50 mM, pH 6.8; ICN, Costa Mesa, CA) allowed to maintain a constant pH throughout the growth of this acid-producing *Rhizobium sp.* (Jarvis *et al.* 1982). When used, agar (1.5%, w/v) was added to either the complex or the defined media.

D.2.3. Cosmid Purification and *Rhizobium* Transformation

E. coli cells containing pRmM57 were used as a source of cosmid DNA for *nodC::lacZ* fusions. *R. loti* cells containing p2112 were used as a source of Flavonoid-Independent Transcriptional Activator *nodD* (FITA *nodD*) containing cosmid as a positive control in the biosynthesis and accumulation studies.

All cosmid purifications were achieved using the alkaline lysis method described by Sambrook *et al.* (1989). Briefly, the lysate was centrifuged (12,000 rpm, 10 min., 4°C), the resuspended pellet washed with equilibrated phenol-chloroform (1:1, v/v, pH 8.0), and centrifuged (12,000 rpm, 10 min, 4°C). The aqueous phase was precipitated with 2 volumes of absolute EtOH (-20°C), centrifuged, and the lyophilyzed pellet resuspended in TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA). The latter extract was treated with DNase-free RNase at 37°C until the preparation was free of RNA, and subjected to Proteinase K digestion (37°C, 1 h). This digest was washed twice with phenol-chloroform-*iso*-AmOH (25:24:1, v/v/v), before precipitation with 0.6 vol of *iso*-PrOH and 0.1 vol of 6 M NaOAc at room temperature. The nucleic acid pellet was washed with 70% EtOH to remove any remaining salts, lyophilyzed and resuspended in 0.5 mL of TE buffer.

The transformation of competent *Rhizobium lupini* cells was achieved using the freeze-thaw method (Hofgen and Willmitzer, 1988), which consisted of successive incubations in TE buffer, for 5 min at 4°C, 5 min in liquid N_2 and 5 min at 37°C. *Rhizobium* transformation mixtures were grown in rescue medium (20 mM glucose, 5 mM $MgCl_2$, 2% bacto-tryptone, 0.5% yeast extract, 0.05% NaCl; Sambrook *et al.* 1989) for 6 h, and plated on a selective yeast-mannitol-agar containing 50 $\mu g \cdot mL^{-1}$ spectinomycin. Resistance to tetracyclin (50 $\mu g \cdot mL^{-1}$) was also tested with pRmM57 transformants.

D.3. *IN VITRO* ADSORPTION OF *LUPINUS ALBUS* ISOFLAVONOIDS ONTO *R. LUPINI* MEMBRANES

D.3.1 Experimental Procedure

Cultures of *R. lupini,* or of other *Rhizobium* species, were grown in MA medium (D.2.2) then treated at mid-log phase (ca. OD_{600}=0.4) with 10 μM of each of the indicated flavonoids for 4 h at 28°C. The treated cultures were then placed on ice, centrifuged (10,000 rpm, 10 min, 4°C) and processed following the method of Hubac *et al.* (1993) with slight modification. Briefly, the cell pellets were resuspended in a 0.9% NaCl solution and centrifuged (10,000 rpm, 10 min, 4°C); this procedure was repeated twice. The thoroughly washed cells were resuspended in a minimal volume of 50% aq. EtOH, and boiled for 10 min in a water bath. The residual EtOH was evaporated, and the aq. mixture acidified to pH 4.5, then extracted twice with EtOAc. The combined organic layers were lyophilyzed and resuspended in a minimal volume of 80% aq. MeOH for HPLC analysis. The bacterial supernatants, as well as the NaCl washings, were also extracted to recover the unadsorbed flavonoids during EtOAc extraction and analyzed by HPLC as described above. HPLC analyses were carried out in an isocratic-mode of elution (ca. 75, 90 or 95% of aq. MeOH) depending on the polarity of the flavonoids used with each treatment (see Table 4).

D.3.2. Data Processing

In order to calculate the rate of adsorption of flavonoids (nmol·mg^{-1} bacterial protein), a correlation was established between the OD_{600} values of *Rhizobium* cultures and their protein content. Extinction coefficients of flavonoids at 280 nm, determined in an isocratic mode of HPLC analysis, were also established (Table 4). The amounts of adsorbed flavonoids were calculated from the protein content and the extinction coefficients of flavonoids according to Equation 1 (Figure 5). A database was developed for this

Table 4. Physicochemical characteristics of lupin isoflavonoids and other flavonoids

Compound[1]	Extinction coefficient[2] ($nmol \cdot sec^{-1} \cdot V^{-1}$)	Maximum absorbance[3] (nm)	Molecular weight ($g \cdot mol^{-1}$)	Gradient[4] HPLC (Rt, min)	Isocratic[5] HPLC (% MeOH)	Isocratic[5] Rt (min)
Genistein	0.97	260.6	270.23	14.8	75 %	4.01
2'-Hydroxygenistein	0.77	259.0	286.23	11.7	75 %	3.24
Lupinalbin A	0.71	256.0	285.23	19.7	90 %	3.59
Wighteone	1.24	267.0	338.23	22.3	95 %	3.13
Isowighteone	1.51	262.3	338.23	22.7	95 %	3.19
Lupiwighteone	2.21	265.8	338.23	23.1	95 %	3.27
Derrone	1.81	264.4	338.21	24.3	95 %	3.76
Licoisoflavone A	1.00	261.6	354.23	20.7	90 %	3.12
Luteone	0.94	264.4	354.23	20.4	90 %	3.05
Lupalbigenin	1.47	267.6	406.23	26.1	95 %	3.81
2'-Hydroxylupalbigenin	1.43	267.8	422.23	25.4	95 %	3.52
Prunetin	0.35	263.4	275.43	20.1	90 %	3.52
Isoprunetin	1.03	264.0	275.43	12.1	75 %	3.53
Biochanin A	1.15	265.2	275.43	21.4	90 %	3.57
Luteolin	0.48	267.8*	286.23	14.5	75 %	3.91
Daidzein	0.68	249.4	252.23	12.5	75 %	3.46
Apigenin	0.47	267.3*	270.23	17.6	75 %	4.61
Naringenin	0.57	289.0*	270.23	13.6	ND	ND
7,4'-dihydroxyflavone	0.39	268.7*	252.23	17.8	ND	ND

[1]Structural formulae of isoflavones are shown in Figure 2.

[2]Relative extinction coefficients were determined from duplicate HPLC injections at different concentrations (section D.4.3). Units are shown in $nmol \cdot second^{-1} \cdot volt^{-1}$, at 280 nm, as detected with a 991M photodiode array detector (Waters™).

[3]Values in nanometers (nm) are derived from UV spectra. Individual compounds were dissolved in 80% aq. MeOH and readings were taken in a 1.5 mL quartz cuvette with a 1.0 cm path length.

[4]Retention times of flavonoid standards in gradient HPLC mode (section D.5.2).

[5]Retention times of flavonoid standards in isocratic HPLC mode at the specified MeOH concentration; ND, not determined.

*Another maximum absorbance is observed at longer wavelenghts (ca. 330 to 370nm).

A. Calculation of amounts adsorbed of flavonoid onto bacterial membranes

Eq. 1: $$X\ (\text{nmoles} \cdot \text{mg protein}^{-1}) = \frac{A \cdot \varepsilon \cdot df}{V \cdot OD_{600} \cdot pc}$$

Where: A = surface area of the peak at 280 nm (volts/sec),
ε = extinction coefficient of the identified flavonoid (see Table 4),
df = dilution factor of extract injected into the HPLC
V = volume of culture (5 mL)
OD_{600} = absorbance of cell cultures
pc = protein content for an OD_{600} of 1.0 (mg)

N.B. V, OD_{600} and pc were omitted in calculating the unbound fraction.

B. Calculation of specific growth rates and doubling times

Eq. 2: $X = X_0 + e^{kt}$ thus, $k = (\ln X - \ln X_0) / t$ **Eq. 3:** $G = \ln 2 / k$

Where: X = OD_{600} during growth,
X_0 = OD_{600} at the beginning of assay,
t = time of incubation (h),
G = doubling time (h),

C. Calculation of relative ß-galactosidase activity

Eq. 4: $$U\ (\text{units of ß-galactosidase}) = \frac{1000 \cdot OD_{420}}{OD_{600} \cdot t \cdot V}$$

Where: OD_{600} = absorbance of cultures at the end of the induction period
OD_{420} = absorbance at the end of the assay due to colour formation
t = duration of assay
V = volume of induced cells used for the ß-galactosidase assays

Figure 5. Equations employed for various calculations

analysis and processed with the EXCEL™ software.

D.4. GROWTH OF *R. LUPINI* IN PRESENCE OF ISOFLAVONOIDS

D.4.1. Experimental Procedure

Triplicate cultures originating from single colonies were grown to mid-log phase in either YM, MA or GS liquid media (section D.2.2). The bacteria were diluted in the same medium to an OD_{600} of 0.1, and 4-mL aliquots were dispensed into sterile test tubes (150 x 25 mm I.D.). Fourty µL of the test isoflavonoids solubilized in 80% aq. MeOH were added to the cultures, where the final MeOH concentration was 0.08%. Control curves were established with 80% aq. MeOH for flavonoids, and with water for aldonic acids. Each of the test compounds (Table 1) was assayed at different concentrations. The different growth assay mixtures were incubated at 28°C (22°C for *Agrobacterium rhizogenes*) on a rotaty shaker at 300 rpm. Growth rates were monitored by measuring the OD_{600} of a $1/5^{th}$ dilution of each culture; and eight data points per strain, per treatment were used to generate each of the triplicate growth curves.

D.4.2. Data Processing and Statistical Analysis

A database was developed for each strain and processed with the EXCEL™ software. A specific growth rate constant (k) was calculated from the logarithmic portion of each triplicate growth curve according to Equation 2 (Figure 5; Neidhart *et al.* 1990). Regression coefficients were calculated as a measure of confidence in k determinations; and doubling times (G) were calculated according to Equation 3 (Figure 5; Neidhart *et al.* 1990).

To determine if the variations among the specific growth rates were statistically significant, the triplicate G values were tested with a one-way ANOVA ($P \geq 0.95$),

followed by a Student-Newman-Keuls (SNK) range test using the NCSS™ software (NCSS, 1990).

D.5. *NOD* GENE INDUCIBILITY WITH *NodC::LacZ* FUSIONS

D.5.1. Preparation of *L. Albus* Seed Effusates and Root Exudates

Seeds of white lupin, *Lupinus albus* cv. Kievskij and Primovsky, were surface sterilized by flaming in EtOH for 10 sec, then shaking with a water-bleach solution (4:1, v/v) containing 0.05% Tween 20 on a rotary shaker at 250 rpm for 30 min, followed by thorough rinsing with sterile water. Surface sterilized seeds were allowed to imbibe water for 24 h while shaking (175 rpm) under aseptic conditions. Following imbibition (t = 24 h), the seeds were placed with the emerging roots facing down on sterilized vermiculite in polycarbonate containers under aseptic conditions, and incubated under the following conditions: 16-h light (ca 400 $E \cdot m^{-2} \cdot sec^{-1}$) and 8-h dark, at 26°/20°C, and 50% relative humidity. At t = 48 h, the seedlings with straight emerging roots (10 to 25 mm) were mounted aseptically on stainless steel grids placed over 300 mL beakers containing nutrient media inside the polycarbonate boxes (Gagnon, 1993). This growth medium, which was prepared with deionized water and filter-sterilized prior to use, consisted of a nutrient solution in which the N-containing salts were replaced with N-free salts, and adjusted to pH 6.5 (Johnson *et al.* 1957; Gagnon, 1993).

Seed effusates and root exudates were adjusted to pH 4.5, before being extracted twice with an equivalent volume of EtOAc, and the combined organic layers were evaporated at 40°C under vacuum. The residue was redissolved first with 80%, followed by 100% MeOH, and the combined extracts were lyophilyzed and redissolved in a measured volume of 80% aq. MeOH.

D.5.2. HPLC Fractionation of Lupin Seed Effusates and Root Exudates

HPLC analysis of seed effusates and root exudates was carried out at a flow rate of 1 $mL \cdot min^{-1}$ using 45% solvent A (0.5% methanolic HOAc) in 55% solvent B (0.5% aq. HOAc) for 2 min, followed by a gradient increase to 100% solvent A in 23 min. Sixty-drop fractions (ca. 1.5 mL) from HPLC eluates were collected in Eppendorf tubes, correlated to precise retention times, and lyophilyzed in a Speed-Vac concentrator. These seed effusate and root exudate fractions were used as induction mixtures to treat *R. lupini*/pRmM57 transformants (section D.2.3).

D.5.3. ß-Galactosidase Assays with *R. lupini*/pRmM57 and *R. meliloti*/pRmM57

ß-Galactosidase assays were carried out according to Sambrook *et al.* (1989), but with slight modification. Briefly, the transformed strains were induced with isoflavone standards, or with HPLC-purified seed effusates or root exudates (D.5.2), and grown from OD_{600} of 0.1 to 0.6 in YM medium for 4 h (*R. meliloti*) or 18 h (*R. lupini*). One hundred µL-aliquots of these cultures were mixed with 900 µL of Z buffer (0.1 M KH_2PO_4, 0.01 M $MgSO_4$, 0.01 M KCl, pH 6.8) containing 30 µL of 0.5% (w/v) sodium dodecyl sulfate and 30 µL of chloroform. A 200 µL aliquot of *o*-nitrophenyl galactoside (ONPG, 4 $mg \cdot mL^{-1}$; w/v) was added to the assay mixture prior to incubation at 28°C. At the end of the assay, 250 µL of 2.5 M sodium carbonate was added to the reaction mixture, centrifuged, and ß-galactosidase activity was monitored at 420 nm. The relative ß-galactosidase activities, expressed in Miller units (U), were calculated according to Equation 4 (Figure 5).

D. 6. RADIOLABELING AND ACCUMULATION OF *NOD* METABOLITES (LCOs)

D.6.1. Radiolabeling of *Nod* Metabolites and TLC Analysis

Mid-log phase cultures of *R. lupini,* or of other *Rhizobium* species, grown in YM or GM media were diluted to an OD_{600} of 0.1 and 6-mL aliquots dispensed into test tubes.

The latter were treated for 3 h (Laeremans *et al.* 1996) with flavonoids and/or aldonic acids in the case of wild-type strains, or with naringenin for the p2112 transformants. The induced cultures were then administered either 1.25 or 2.8 μCi of [1-^{14}C] sodium acetate, or 0.5 μCi of either D-[1-^{14}C]glucosamine, -galactosamine, -mannosamine, -acetylglucosamine or -acetylgalactosamine, and allowed to take up the radiolabeled precursors for 18 h (Laeremans *et al.* 1996) or 40 h. At the end of the metabolic period, the test cultures were centrifuged in a clinical centrifuge (5000 rpm, 90 min), and the supernatants passed through a C18 RP Sep-Pack™ pre-equilibrated with water, and washed with 10 mL of water. The labeled LCOs were then eluted with 5 mL of absolute MeOH (Laeremans *et al.* 1996), lyophilized, resuspended in 80% of aq. MeOH and applied onto HPTLC plates (Spaink *et al.* 1992; Laeremans *et al.* 1996). The latter were chromatographed with water-acetonitrile (50:50, v/v; Spaink *et al.* 1992), and autoradiographed with X-Omat AR film (Kodak, Rochester, NY). The films were developed after a period of time corresponding to the amount of radioactivity loaded on the plates (ca. 4- to 20-d exposure). In some cases, TLC plates were placed in a Molecular Imager™ (Bio-Rad, Mississauga, ON) for a shorther period of time (ca. 2-4 d). In order to assess the identity of the inducible radiolabeled LCOs, the spots were scraped off the TLC plates, and 2x-extracted with 80% and 100% MeOH. The combined extracts were lyophilized and resuspended in a minimal vol. of 80% aq. MeOH or water, for either HPLC gradient analysis (section D.6.2), or enzymatic characterization of *Nod* factors (section D.6.3), respectively.

The radiolabeled bacterial pellets were extracted overnight with a single phase Bligh-Dyer mixture (chloroform-methanol-50mM phosphate buffer in 150 mM NaCl, pH 7.5; 1:2:0.8, v/v/v). The extraction mixture was centrifuged (10,000 rpm for 2 min) to remove the cell debris, and was converted to a two-phase extract by the addition of equivalent volumes of chloroform and buffer (Price and Carlson, 1995). The organic phase was then recovered and lyophilized for radioactivity determination.

In addition, all plasticware (microfuge tubes, tips etc.) used for LCO extractions (whether radiolabeled or not) was silanized with dimethylchlorosilane (VWR Canlab, Ville Mont-Royal, QC) and washed with distilled water, in order to eliminate any possible adsorption of LCOs (Spaink *et al.* 1992).

D.6.2. HPLC Analysis of LCOs

The analytical HPLC gradient-elution protocol of radiolabeled LCOs consisted of 2 min in 15% aq. AcN, followed by an increase to 40% aq. AcN in 13 min, and to 100% AcN in 10 min. The HPLC eluates were monitored at 206 nm, and 750 µL fractions were collected for liquid scintillation counting.

D.6.3. Hydrolysis of *Nod* Factors by Specific Enzymes

The TLC-separated, radiolabeled *Nod* factors (D.6.1) were resuspended in 30 µL water, divided into 5-µL aliquots and subjected to hydrolysis with one of the following enzymes: 250 U of porcine kidney acylase I, in 100 mM phosphate buffer, 120 mM NaCl, pH 7.0; 1.0 U of egg white lyzozyme, in 100 mM Tris-HCl, pH 6.8; 0.1 U of chitinase from *S. marcescens*, in 50 mM citrate, 100 mM potassium phosphate, pH 6.0; 0.25 U of *N*-acetylglucosaminidase from jack beans, in 50 mM citrate, pH 5.0; and 0.2 U of Proteinase K, in 10 mM Tris HCl, pH 7.5. The enzyme reaction mixtures were incubated for 24 h at 30°C and the hydrolysates were lyophilyzed, resuspended in 80% aq. MeOH and loaded onto HPTLC plates. The latter were chromatographed by HPLC (D.6.2), or TLC for three succecive runs in 50% aq. AcN, and autoradiographed.

D.6.4. LCO Accumulation *In Vitro*

R. lupini and *R. loti* cultures were grown in 30 mL of YM medium, diluted to an OD_{600} of 0.1 before being treated with either flavonoids or aldonic acids for 21 h, for the production of *Nod* factors. The treated cultures were centrifuged (10,000 rpm, 10 min,

4°C), and the supernatants were pre-purified and lyophilized as described in section D.6.1. The latter were resuspended in a minimal volume of 80% aq. MeOH for analytical HPLC analysis (section D.6.2). The putative active fractions, based on their spectral similarities to LCOs, were used for the root hair deformation (*Had*) bioassay with *L. albus* seedlings.

D.7. ROOT HAIR DEFORMATION ASSAYS

Lupinus albus seeds were germinated as described in section D.5.1. until the secondary roots emerged (i.e. 7d). Three root segments, harboring root hairs, were excised and placed, three roots per well, onto sterile 24-well plates. One-mL aliquots of water containing various dilutions of the HPLC-purified LCO fractions (extracted from induced *R. loti*/p2112 or wild type *R. lupini*), were applied onto each of the root segments. Dilute methylene blue (0.01%, w/v) was added in order to stain the root hairs and epidermal cells. Root segments were visualized with a dissecting microscope equipped with a 40 X objective, and root hair deformation was monitored 18 h after treatment.

D.8. ALDONIC ACIDS IN LUPIN SEEDS, SEED EFFUSATES AND ROOT EXUDATES

Aldonic acids were extracted from seed effusates and root exudates as described in section D.5.1. In addition, seed effusates were also pre-purified on C18 Sep-Pack™ by successive elution with 50% and 100% MeOH, followed by concentration and resuspension of these eluates in 50% and 80% aq. MeOH, respectively. Cotyledons and seed coats (24, 48, 72 and 96 h old) were ground with a glass rod in an microfuge tube, extracted twice with 80% aq. MeOH and once with 100% MeOH; the combined extracts were lyophilyzed and resuspended in a minimal volume of 80% aq. MeOH.

These extracts were analyzed by gradient-HPLC using a semi-preparative RP-C18 column, under the following conditions: 2 min in 5% methanolic phosphoric acid (pH 3), followed by an increase to 100% MeOH in 15 min, and maintained for a further 10 min. UV absorbance was recorded between 200 to 400 nm during elution, and peak areas were integrated based on their absorbance at 220 nm..

E. RESULTS

E.1. GENERAL COMMENTS AND OBSERVATIONS

E.1.1. Quantitative Analysis by HPLC

In order to achieve the objectives outlined in section B, various high pressure liquid chromatography (HPLC) protocols were developed for both qualitative and quantitative analyses of extracts containing either flavonoids, aldonic acids or LCOs. The extinction coefficients deduced from the calibration curves of isoflavones at 280 nm are shown in Table 4, whereas those of erythronic and tetronic acids, measured at 220 nm, amounted to 1.976 and 1.059 $\mu mol \cdot volts^{-1} \cdot sec^{-1}$, respectively. LCOs were not calibrated.

The choice of elution solvents for HPLC analysis was the result of a compromise between solvent cost and its UV cut-off. In fact, LCOs which have a maximum absorbance (λmax) at 206 nm required for analysis the more expensive, low UV cut-off AcN (Spaink *et al.* 1992), whereas the λmax of isoflavones (which varies between 250 and 280 nm) and that of aldonic acids (ca. 220 nm) allowed the use of a cheaper, high UV cut-off solvent such as MeOH. It is well known that HPLC analysis on C18 supports provides a good fractionation of most flavonoids (Graham, 1991), of lupin isoflavonoid aglycones and their derivatives (Gagnon *et al.* 1992a), as well as of LCOs (Lopez-Lara *et al.* 1995). For aldonic acids, however, no HPLC protocol currently exists and, therefore, one had to be developed.

E.1.2. Growth Media Formulations for *Rhizobium lupini*

Minimal media formulations that are known to support the growth of other *Rhizobium spp.* (Recourt *et al.* 1989; Hartwig *et al.* 1991) do not sustain that of *R. lupini*. In fact, the *in vitro* cultivation of the acid-producing *R. lupini* (Jarvis *et al.* 1982) in minimal media leads to acidification of the culture medium to pH 4.6; thus resulting in a delayed bacterial

growth and poor experimental conditions for flavonoid or LCO analyses (data not shown). Therefore, buffering of the medium with MOPS was necessary to maintain a relatively constant pH. Using the different culture media formulations outlined in section D.2.2., the optimal growth of *R. lupini* was achieved at 28°C, with doubling times of ca. 34.6, 15.7, 13.5 and 8 h in the MA, GS, GM and YM media, respectively. The ammonium chloride concentration in the MA medium was reduced to 0.01%, since higher concentrations are known to impair *nod* gene inducibility. In fact, whereas low concentrations of nitrogen salts are required for optimal *nod* gene induction and *Nod* factor accumulation (McKay and Djordjevic, 1993), those of succinate and glutamate are not (Poupot *et al.* 1993). Furthermore, polymyxinB sulfate (colistin sulfate) was added to both solid and liquid media as a selection marker, since *R. lupini* is naturally resistant to this antibiotic (Pankhurst, 1977).

E.1.3. Physicochemical Characteristics of Aldonic Acids

In contrast with erythronic acid, the pH of concentrated (ca. 1M) standard solutions of tetronic acid is very low (ca. pH 2.9). However, tetronic acid cannot be buffered, since it exists in either strongly acidic or basic forms (data not shown). In addition, whereas the addition of erythronic acid does not affect the pH of the growth media, tetronic acid leads to extensive acidification, ranging from 0.1 pH unit at 10 mM to 1.2 pH unit at 15 mM concentration, with an apparent exponential shift (data not shown). However, the addition of 10 mM of either of the aldonic acids to non-inoculated growth media, leads to a minor change in OD_{600} (ca. 0.001 unit).

E.2. ADSORPTION OF FLAVONOIDS ONTO *RHIZOBIUM* CELL MEMBRANES

E.2.1. Qualitative and Quantitative Analyses

The *in vitro* adsorption of known flavonoid inducers onto the membranes of compatible *Rhizobium sp.*, as well as that of lupin isoflavones onto *R. lupini* membranes, was determined with bacterial cultures grown in MA medium, after a 4-h incubation in the presence of 10 μM of the test compounds. Duplicate adsorption assays were carried out for each compound, and each test extract was subjected twice to HPLC analysis, resulting in four values whose averages are reported in Tables 5 and 6. In order to quantify the amounts of flavonoids in the different fractions analyzed, a calibration curve between OD_{600} values and protein contents was first established for each bacterial species cultivated in the MA medium. For example, the amount of *R. lupini* protein determined for an OD_{600} of 1.0 was 52.4 μg protein·mL^{-1}, whereas these values were 25.41, 37.12 and 48.78 μg protein·mL^{-1} for *Bradyrhizobium japonicum*, *R. leg.* bv *viciae* and *R. meliloti*, respectively. The isocratic HPLC conditions used for the determination of the different flavonoids tested, their retention times under these conditions, as well as their extinction coefficients calibrated at 280 nm are reported in Table 4. The adsorption values presented in Tables 5 and 6 were calculated according to Eq. 3 (Figure 5), taking into account the surface area of the peak of interest, the total amount of protein and the amount of extract analyzed. The results of the adsorption assays for various *Rhizobium spp.* are reported in Table 5 and those for *R. lupini* in Table 6.

E.2.2. Adsorption of *Nod* Gene Inducers onto *Rhizobium* Cell Membranes

Adsorption assays carried out with *R. meliloti* resulted in an adsorption onto the cell membranes which amounted to 7.8 nmol·mg^{-1} bacterial protein of the known inducer, luteolin (Table 5). This value is similar to that reported for the latter symbiotic association (ca. 11 nmol·mg^{-1} protein in TY medium; Hubac *et al.* 1993), and confirms that the assay

Table 5. *In vitro* adsorption of flavonoids onto cell membranes of different rhizobia[1]

Flavonoid tested	Bound fraction[2]	Standard deviation[3] (%)	Unbound fraction[4]	Standard deviation (%)	Recovery[5] (%)
Rhizobium meliloti					
Luteolin	7.8	10	13.8	16	32
Luteolin (with genistein)	5.6	12	19.4	14	39
Bradhyrhizobium japonicum					
Daidzein	11.9	*	15.5	21	42
Genistein	3.5	17	37.4	15	75
Rhizobium leguminosarum* bv *viciae					
Apigenin	7.4	15	19.0	*	38
Luteolin	1.8	*	15.9	7	32

[1]Duplicate *Rhizobium* cultures grown in MA medium (OD_{600} 0.4), were administered flavonoids at a final concentration of 10 μM for 4 h, prior to centrifugation and washing with NaCl. Control cultures were administered 80% aq. MeOH.

[2]Values represent averages of 4 HPLC determinations (i.e. duplicate experiments, each of which were analyzed twice by HPLC), in nmol of bound flavonoid per mg bacterial protein, as determined after analysis of the boiled cell-pellets.

[3]Standard error (% of SE) of averaged HPLC determinations; *, SE<5%.

[4]Total of unbound flavonoid in nmol (averaged as in [2]), as determined after HPLC analysis of the ethyl acetate-extracted culture supernatants.

[5]Percent recovery of the original amount of flavonoid administered (i.e. 50 nmol). This includes the bound and unbound fraction.

Table 6. *In vitro* adsorption of isoflavonoids onto *Rhizobium lupini* cell membranes[1]

Isoflavonoid tested	Bound fraction[2]	Standard deviation[3] (%)	Unbound fraction[4]	Standard deviation (%)	Recovery[5] (%)
Genistein	4.99	*	27.2	6	54
2'-Hydroxygenistein	2.34	23	47.2	*	94
LupinalbinA	114.9	*	19.2	29	38
Luteone	55.1	32	20.1	6	40
LicoisoflavoneA	25.2	21	17.9	7	36
Wighteone	320.4	10	35.8	14	72
Isowighteone	130.3	12	19.8	10	90
Lupiwighteone	206.8	11	13.7	10	28
2'-Hydroxylupalbigenin	40.6	25	4.22	13	8
Lupalbigenin	92.3	17	5.46	15	11
Derrone	378.7	8	24.5	9	49
Isoprunetin	27.5	22	36.9	22	74
Prunetin	145.6	7	30.2	49	60
Biochanin A	25.9	*	20.5	30	41

[1 to 5] as in Table 5.

conditions were optimum. When *R. meliloti* was incubated in the presence of a mixture of the inducer and inhibitor molecules (i.e. luteolin and genistein), the amount of luteolin adsorbed was found to be 5.6 nmol·mg^{-1} protein, which contrasts with that obtained with the inducer alone. In addition, the amount of daidzein adsorbed onto the membranes of *Bradyrhizobium japonicum* was found to be greater than that of a minor *nod* gene inducer, genistein (Table 5). The same phenomenon was observed with *Rhizobium leguminosarum* bv *viciae,* where apigenin (a major inducer) is adsorbed at a relatively higher rate than luteolin (Table 5).

E.2.3. Adsorption of Lupin Isoflavones onto *Rhizobium lupini* Cell Membranes

The amounts of lupin isoflavones adsorbed onto *R. lupini* membranes were found to vary between ca. 2.3 nmol for 2'-hydroxygenistein to 380 nmol for derrone·mg^{-1} protein (Table 6). Samples of the elution profiles of these extracts, shown in Figure 6, demonstrate the extensive range of the adsorption levels observed. It should be noted, however, that the elution profile of the control (80 % MeOH treatment) exhibits several peaks (Figure 6A), but at a scale of very low magnitude, as compared to those of the different isoflavone treatments. Of all the isoflavones tested, the highest adsorption levels observed were, in a decreasing order, derrone > wighteone > lupiwighteone > prunetin ≥ isowighteone > lupinalbin A > lupalbigenin. It appears, therefore, that the levels of isoflavone adsorption are higher for the monoprenylated derivatives of genistein than for the diprenyls, e.g. lupalbigenin (Table 6). In addition, whereas the observed rates of isoflavone adsorption are inversly related to the level of isoflavone prenylation, the presence of a 2'-hydroxyl substituent appears to reduce the adsorption rate of isoflavones (e.g. lupalbigenin > 2'-hydroxylupalbigenin; wighteone > luteone; Table 6).

Although the amounts of unbound isoflavones extracted from the culture supernatants reflect the level of adsorption for most isoflavone treatments, the percentages of isoflavones recovered, relative to the administered amounts, are quite low in some treatments as

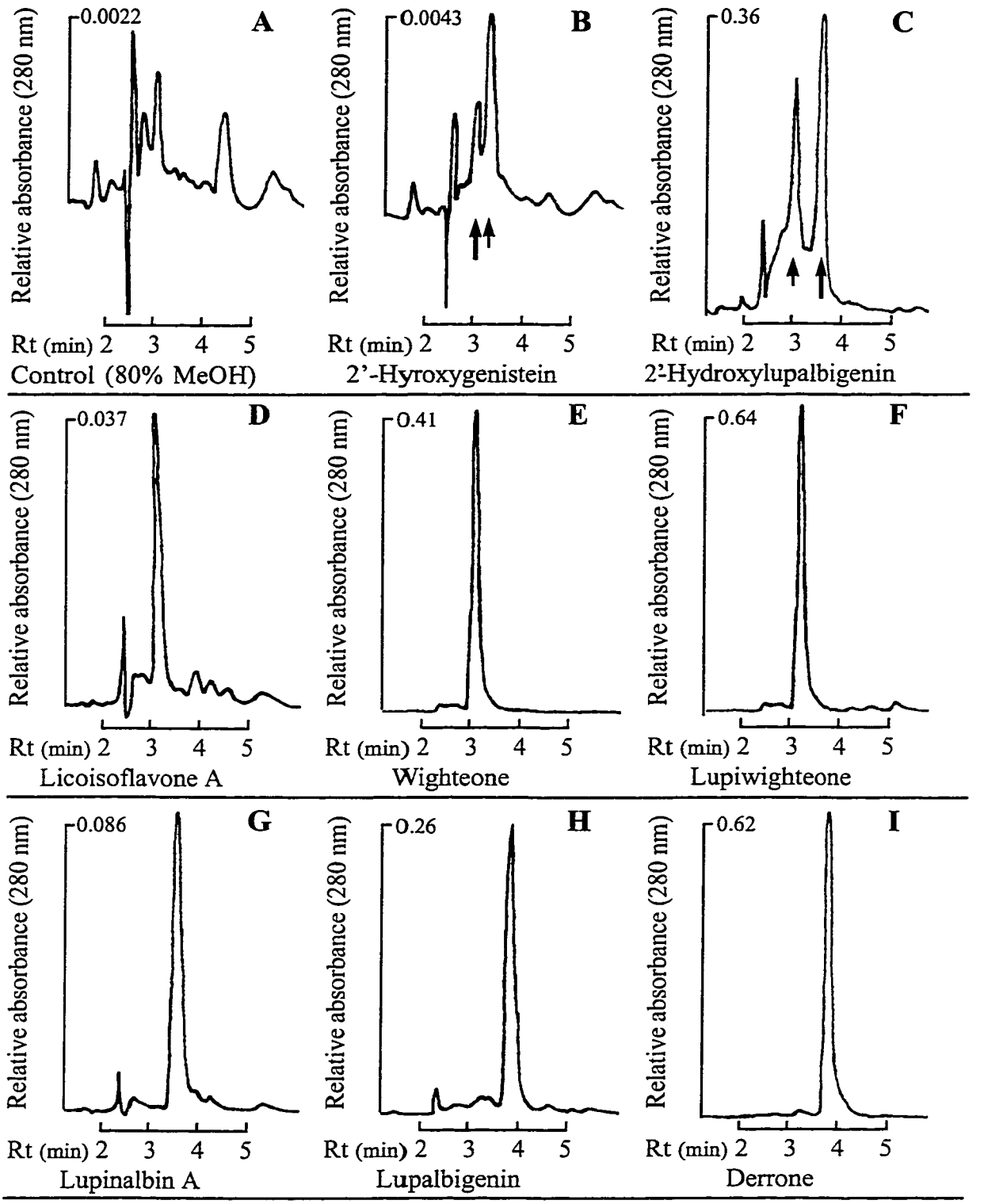


Figure 6. HPLC profiles of isoflavonoids adsorbed onto *Rhizobium lupini* membranes. Chromatograms were scaled to highest peak, with a maximum relative absorbance indicated on top left of each panel. Identified isoflavonoids are indicated at bottom of panel, except in panels B and C, where the small and big arrows indicate unidentified and identified peaks, respectively.

compared to others (Table 6). The fact that, the percentage recovery of diprenylated compounds was the lowest among all of the isoflavones tested suggests their complexation with other macromolecules. This is corroborated by the immunocytolocalization of 2'-hydroxylupalbigenin with bacteroid DNA (Gagnon *et al.* 1995a). Furthermore, the relatively low level of unbound lupiwighteone and its low % recovery are remarkable, as compared with those of other aglycones, including the monoprenylated- and methylated derivatives. Additional peaks were observed after HPLC analysis of cell pellets treated with 2'-hydroxylupalbigenin (Figure 6B) and, to a lesser extent, with 2'-hydroxygenistein (Figure 6C).

E.3. EFFECT OF VARIOUS COMPOUNDS ON GROWTH RATES OF RHIZOBIA

E.3.1. Growth of *R. lupini* and of Other Rhizobia

General Considerations

The effects of *L. albus* isoflavones and of synthetic aldonic acids on the *in vitro* growth rates of different rhizobia have been investigated using three different growth media, the mannitol ammonium chloride (MA), the glutamate succinate (GS) and the yeast mannitol (YM) media (D.2.2). Growth curves were established from triplicate bacterial cultures for each isoflavone treatment and, following processing, the lowest regression coefficient calculated for the growth-rate constants amounted to 0.90. The calculated range of doubling times used in the SNK test were 3.26 h, 1.32 h and 0.39 h for *R. lupini* grown in MA, GS and YM media, respectively; whereas those for *R. meliloti* and *A. rhizogenes* growth in YM medium were 0.51 h and 1.22 h, respectively. An aliquot of each of the triplicate cultures was plated onto a Petri dish to assess for contamination.

E.3.2. Effect of Lupin Isoflavones on Growth of *R. lupini* in Mannitol-Ammonium Chloride Medium

The effect of lupin isoflavonoids on the *in vitro* growth rate of *R. lupini* in MA medium was tested at two different concentrations (2.5 and 10 μM). The results of these growth

tests are reported in Table 7, and the growth curves for the different treatments are shown in Appendix I. Of the various isoflavones tested, only a few compounds resulted in a significant growth inhibition at 10 μM, as compared with that of the control (Table 7). Treatment with 10 μM prenylated isoflavonoids resulted in a significant growth inhibition in the following descending order: derrone = 2'-hydroxylupalbigenin ≥ isowighteone ≥ lupalbigenin > control. On the other hand, a significant growth stimulation was observed after treatment with 10 μM of lupinalbin A, which contrasts with the inhibitory effect of the other isoflavonoids tested (Table 7). Since *nod* gene inducers are expected to stimulate the growth rate of the symbiont, lupinalbin A is a putative candidate as a *nod* inducer compound.

E.3.3. Effect of Lupin Isoflavones on Growth of *R. lupini* in Glutamate Succinate Medium

Significant effects have been observed on *R. lupini* growth in GS medium after treatment with lupin isoflavonoids at three different concentrations (0.5, 5 and 10 μM), as compared with the control cultures (Table 8, Appendix II). Except for lupiwighteone and lupalbigenin which did not affect growth at 10 μM, treatments with the same concentrations of prenylated isoflavonoids resulted in a significant growth inhibition in the following descending order: luteone ≥ 2'-hydroxylupalbigenin = wighteone ≥ biochanin A > derrone = isowighteone = genistein ≥ 2'-hydroxygenistein = licosioflavone A = lupinalbin A ≥ control (Table 8). Furthermore, there was a significant growth inhibition observed with 5 μM isoflavones which correlated, although to a lesser extent, with those observed with the 10 μM treatments. For example, whereas treatments with 5 μM luteone or 2'-hydroxylupalbigenin were inhibitory to *R. lupini* growth, these effects were less significant than treatments with 10 μM . In contrast, 0.5 μM treatments with compounds such as derrone, licosioflavone A, lupinalbin A, the genistein monoprenyls (wighteone, lupiwighteone and isowighteone) and lupalbigenin, resulted in a significant growth

Table 7. Effect of various concentrations of *Lupinus albus* isoflavonoids on the growth rate of *Rhizobium lupini* in MA medium[1]

Isoflavonoid tested	2.5 μM			10.0 μM		
	G[2] (h)	SE[3] (%)	SNK[4,5] Test	G (h)	SE (%)	SNK Test
Control[6]	34.6	*	**b**	34.6	*	**b**
Genistein	34.5	9	**b**	34.6	8	**b**
2'-Hydroxygenistein	36.8	6	**b**	36.1	12	**b**
Lupinalbin A	36.2	7	**b**	30.6	*	**a**
Luteone	35.9	9	**b**	36.8	6	**b**
Licoisoflavone A	37.9	*	**b**	37.3	*	**b**
Kievitone	35.0	*	**b**	37.9	12	**b**
Wighteone	34.5	*	**b**	37.8	6	**b**
Isowighteone	35.2	*	**b**	41.9	15	**c,d**
Lupiwighteone	34.8	*	**b**	35.5	*	**b**
2'-Hydroxylupalbigenin	36.3	*	**b**	43.7	8	**d**
Lupalbigenin	35.6	8	**b**	39.9	*	**c**
Derrone	36.0	*	**b**	45.1	7	**d**

[1]Growth of bacteria was monitored (OD_{600}) from triplicate cultures in MA medium.

[2]Averages of triplicate doubling times (G) in h.

[3]Standard errors (% of SE) of averaged doubling times; *, SE<5%.

[4]All triplicate determinations (regression coefficients of growth curves >0.9) were compared using oneway ANOVA, followed by Student-Newman-Keuls range test.

[5]Bold letters denote different homogeneous subsets, whose doubling times are significantly different from each other.

[6]Control cultures were treated with 80% aq. MeOH.

Table 8. Effect of various concentrations of *Lupinus albus* isoflavonoids on the growth rate of *Rhizobium lupini* in GS medium[1]

Isoflavonoid tested	0.5 μM			5.0 μM			10.0 μM		
	G[2] (h)	SE[3] (%)	SNK[4,5] Test	G (h)	SE (%)	SNK Test	G (h)	SE (%)	SNK Test
Control[6]	15.7	6	**b**	15.7	6	**b**	15.7	6	**b**
Genistein	16.2	*	**b**	17.9	*	**b**	19.6	8	**c**
2'-Hydroxygenistein	19.3	7	**c**	18.2	6	**b,c**	18.5	6	**b,c**
Lupinalbin A	13.3	15	**a**	16.3	7	**b**	18.1	8	**b,c**
Luteone	15.3	11	**b**	22.2	9	**d**	24.4	*	**e**
Licoisoflavone A	13.1	*	**a**	16.5	6	**b**	18.3	7	**b,c**
Kievitone	ND	-	-	ND	-	-	17.9	*	**b**
Wighteone	13.3	12	**a**	18.2	*	**b,c**	23.4	*	**d,e**
Isowighteone	13.3	10	**a**	17.2	*	**b**	19.9	*	**c**
Lupiwighteone	13.6	9	**a**	16.3	*	**b**	17.8	7	**b**
2'-Hydroxylupalbigenin	15.6	*	**b**	22.5	8	**d**	24.0	6	**d,e**
Lupalbigenin	13.9	*	**a**	17.6	*	**b**	17.0	*	**b**
Derrone	13.0	*	**a**	19.8	*	**c**	20.8	*	**c**
Prunetin	ND	-	-	ND	-	-	17.2	*	**b**
Isoprunetin	ND	-	-	ND	-	-	17.0	*	**b**
Biochanin A	ND	-	-	ND	-	-	21.2	8	**d**
Luteolin[7]	15.4	6	**b**	25.2	7	**e**	25.8	8	**e**

[1] to [6] as in Table 7.

[7] A flavone, 5,7,3',4'-tetrahydroxyflavone.

stimulation. The latter compounds may appear to be putative *nod* gene inducers, however, the fact that neither lupalbigenin nor lupiwighteone (5 μM or 10 μM) elicited any significant growth inhibition, suggests that these two isoflavones may act as putative *nod* gene inducers.

In contrast with *R. lupini*, no significant effect on the *in vitro* growth rate of *R. meliloti* in GS medium was observed after treatment with genistein, lupinalbin A, luteone, wighteone, isowighteone, lupiwighteone or lupalbigenin (data not shown).

E.3.4. Effect of Aldonic Acids on Growth of Bacteria in Yeast Mannitol Medium

Growth experiments were conducted with non-flavonoid compounds such as erythronic acid and tetronic acid, in order to investigate their effects on the *in vitro* growth rate of *R. lupini*, *R. meliloti*, and *A. rhizogenes* (Table 9, Appendix III). The complex YM medium was used since most bacteria grow well in this medium.

Treatment of *R. lupini*, with 10 mM tetronic acid resulted in a significant growth stimulation which was not observed at lower concentrations. However, erythronic acid was significantly more inhibitory to growth than the strong isoflavone inhibitor, luteone (Table 9A). These effects are in contrast with those observed for *R. meliloti* treated with aldonic acids, which resulted in a significant growth inhibition in the following descending order: isoflavones (e.g. luteone 10 μM) > tetronic acid > erythronic acid > control (Table 9A). The *in vitro* growth rate of *A. rhizogenes* was significantly delayed by 10 μM luteone, and completely inhibited by 10 mM tetronic acid (Table 9, Appendix III). Similar experiments were performed using various concentrations of tetronic acid in order to examine their effect on the growth of *R. lupini* in GS medium. These resulted in a mild, but significant growth stimulation at 10 mM (Table 9B).

Table 9. Effect of various compounds on the growth rate of three rhizobia[1]

Compound tested and concentration	*R. lupini*			*R. meliloti*			*A. rhizogenes*		
	G[2] (h)	SE[3] (%)	SNK[4,5] Test	G (h)	SE (%)	SNK Test	G (h)	SE (%)	SNK Test
A, Yeast-Mannitol medium									
Control[6]	8.04	*	**b**	5.63	*	**a**	3.03	*	**a**
Tetronic acid 10 mM	7.48	*	**a**	6.67	*	**c**	(-)ve	*	**c**
Tetronic acid 1.0 mM	7.88	*	**b**	5.63	*	**a**	3.14	*	**a**
Tetronic acid 100 µM	7.94	*	**b**	5.36	9	**a**	2.97	*	**a**
Erythronic acid 10 mM	9.83	*	**d**	6.22	*	**b**	3.03	*	**a**
Luteone 10 µM	8.39	*	**c**	7.01	*	**d**	26.14	7	**b**
B, Glutamate-Succinate medium									
Control	16.2	6	**b**	ND			ND		
Tetronic acid 10 mM	14.7	*	**a**	ND			ND		
Tetronic acid 1.0 mM	15.3	*	**b**	ND			ND		
Tetronic acid 100 µM	15.8	*	**b**	ND			ND		
Luteone 10 µM	25.7	*	**c**	ND			ND		

[1]Growth of bacteria was monitored (OD_{600}) from triplicate cultures of *Rhizobium* (28°C) and *Agrobacterium* (22°C) in either YM (A) or GS (B) media. Abbreviations: *R., Rhizobium; A. , Agrobacterium;* ND, not determined.

[2 to 6] as in Table 7.

E.4. INDUCIBILITY OF NODULATION GENES IN *RHIZOBIUM LUPINI*

E.4.1. Transformation of *R. lupini* and Preliminary Assays

Purified pRmM57 cosmid was used to transform competent cells of *R. lupini* by a freeze-thaw method. The efficiency of transformation was ca. 60 transformants/μg of cosmid DNA. The transformants were resistant to both spectinomycin and tetracyclin. In addition, a dose-dependent response was observed after treatment with a range of luteolin concentrations (50 nM to 20 μM), as indicated by the net relative ß-galactosidase activity shown in Figure 7. Therefore, it can be concluded that the pRmM57 cosmid is functional in an *R. lupini* background, and that this transformed strain harboring this *NodC::LacZ* fusion can be used in ß-galactosidase assays after treatment with the putative *nod* gene inducers. It should be noted, however, that the transformed cultures used for all subsequent assays were inducible, after treatment with luteolin as indicated by their ß-galactosidase activities (data not shown).

E.4.2. Inducibility of *Nod* Genes in *R. lupini*/pRmM57 after Treatment with HPLC-Fractionated Seed Effusates or Root Exudates

In order to screen for putative isoflavonoid inducers, seed effusates of *L. albus* were fractionated by HPLC gradient elution. Each of the twenty, 1.5 mL fractions was used as an induction mixture with *R. lupini*/pRmM57 cultures. After 20-h induction, duplicate ß-galactosidase assays were carried out as shown in Figure 8. The results show that the fractions eluted at Rt 4 min and 16.5 min (5 seed equivalent) induced higher levels of ß-galactosidase activity which amounted to 54 and 38 Miller units, respectively. Further purification and concentration of the active-fraction peak Rt 16.5 min, revealed spectral properties characteristic of a flavanone, and not one of an isoflavone. Since naringenin, a flavanone inducer compatible with the *nodD1* of *R. meliloti* present in the pRmM57

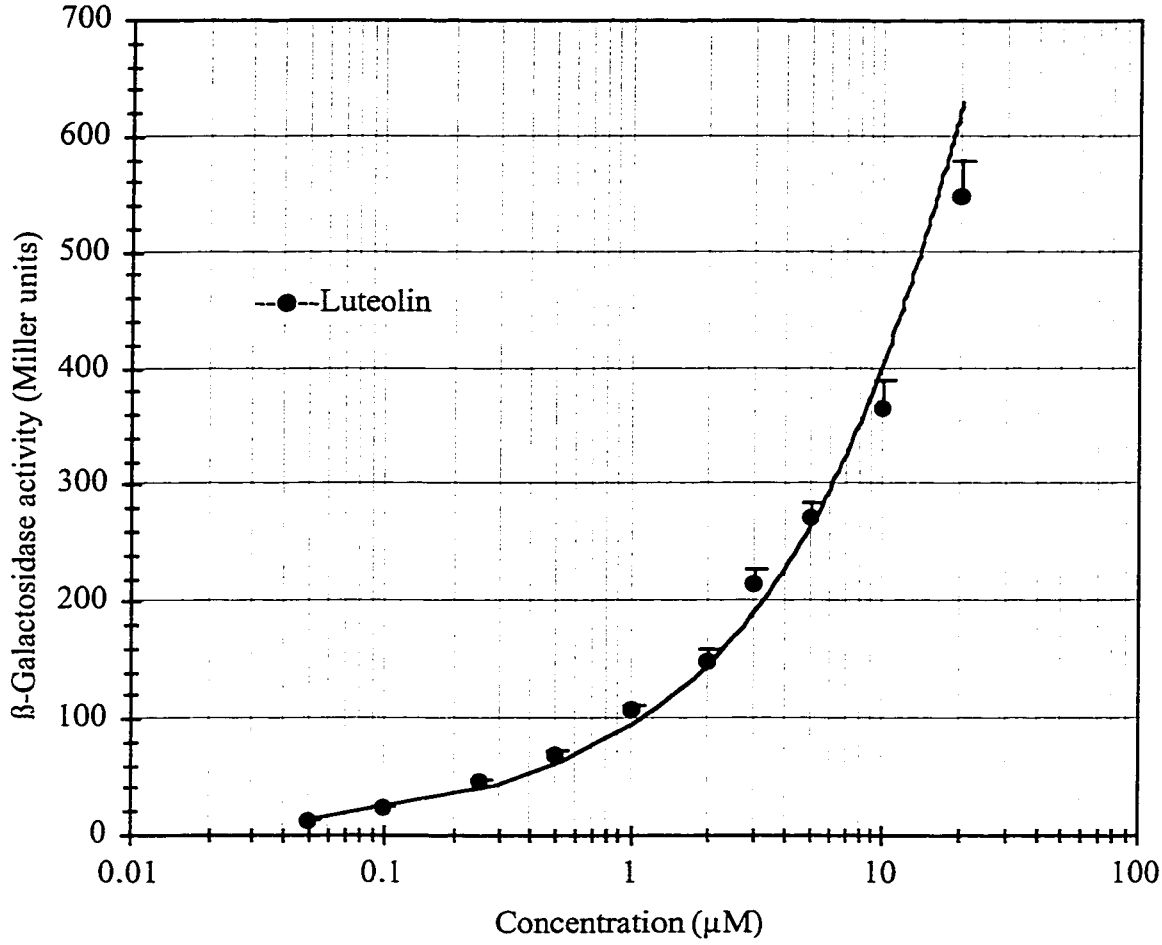


Figure 7. Effect of different luteolin concentrations on ß-galactosidase activity in *R. lupini*/pRmM57

Duplicate *R. lupini*/pRmM57 cultures grown in YM medium, were induced for 20 h with luteolin at various nano- and micromolar concentrations. After induction, triplicate ß-galactosidase assays were carried on each culture. In addition, the ß-galactosidase activity of non-induced controls (i.e. treated with 80% aq. MeOH) was monitored (ca. 18 U). The latter activity was subtracted from those obtained with luteolin treatments to calculate the net values plotted above. Standard errors are indicated by the bars above each plotted value.

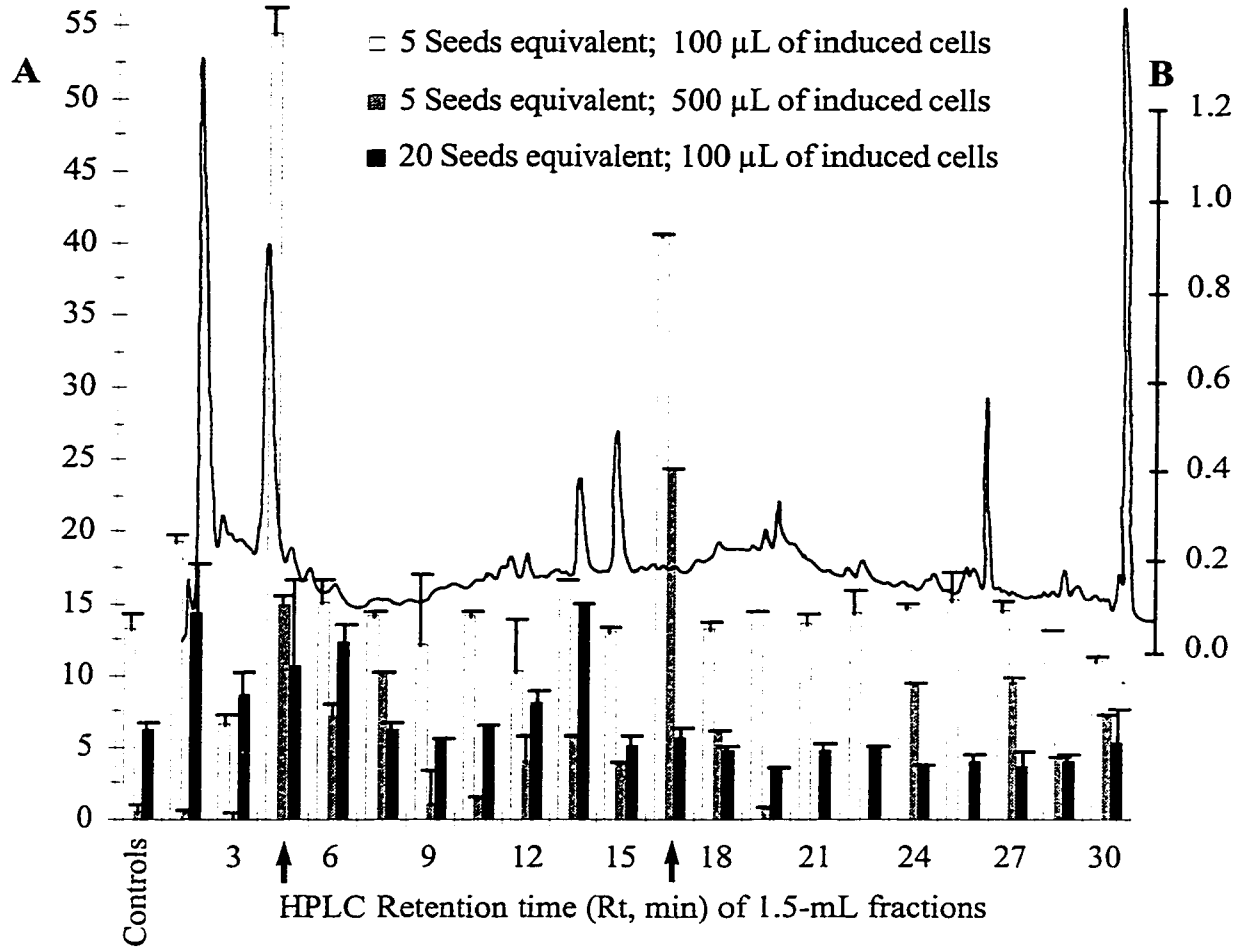


Figure 8. ß-Galactosidase activity of *R. lupini*/pRmM57 cultures treated with HPLC-fractionated seed effusates of *L. albus*

(A) Relative ß-galactosidase activity in Miller units, and (B) relative absorbance at 254 nm of the fractionated seed effusates. Seed effusates were extracted from 24 h-soaked lupin seeds, fractionated by gradient-HPLC (e.g. from 45% to 100% methanolic acetic acid/aq. acetic acid in 23 min; HOAc, 0.5% v/v). Twenty fractions of 1.5-min each were collected and used as induction mixtures for *R. lupini*/pRmM57 grown in YM medium. After a 20-h induction, duplicate ß-galactosidase assays were carried out. Non-fractionated seed effusates gave 16.5 ±0.5 and 14.4±0.3 ß-galactosidase units with either 100 or 500 µL of induced cells, respectively. Treatments were made equivalent to either 5 or 20 HPLC-fractionated seed equivalent. Arrows indicate the most active fractions. Standard errors are indicated by the bars above each plotted value.

cosmid, elutes at this retention time under the same gradient-HPLC conditions used, this activity peak deserves further characterization.

Similar HPLC fractionations were achieved with lupin root exudates, and individual HPLC fractions were used for the induction of *R. lupini*/pRmM57 cultures. ß-Galactosidase assays of the latter fractions showed that none of the 20 fractions tested gave units significantly above those of the controls (Figure 9), suggesting that they do not induce *nod* gene activity.

E.4.3. Inducibility of *Nod* Genes in *R. lupini*/pRmM57 after Treatment with Lupin Isoflavones

Purified isoflavone standards were used at 2, 4, 10 and 20 µM to treat *R. lupini*/pRmM57 cultures, and duplicate ß-galactosidase assays resulted in the relative activities shown in Table 10. None of the isoflavones tested showed any significantly higher ß-galactosidase activity than the controls, except lupalbigenin at 2 µM and derrone at 4 µM. The higher activity observed with 2 µM of 2'-hydroxygenistein 7-*O*-glucoside may be ascribed to the possible metabolism of the sugar moiety by the bacterium, since the aglycone (2'-hydroxygenistein) showed no significant activity. None of the relative ß-galactosidase activities obtained exhibited a dose-dependent response that was proportional to the isoflavone used. In addition, none of the several other lupin isoflavones* tested produced significantly higher ß-galactosidase activitiy than the controls. Therefore, these results suggest that lupin isoflavones, tested alone, can not induce *lacZ* expression in *R. lupini*/pRmM57 and, hence *R. lupini nod* genes.

The possibility that the *nodD1* of *R. meliloti* found in pRmM57 may interfere with the inducing ability of genistein-derived isoflavones, prompted us to delete the *nodD1* gene present in pRmM57. However, none of the isoflavonoids tested (those shown in Table 10)

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* "Topazolin, 3'-*O*-methylorobol, lupiniforiol, alpinum isoflavone, kievitone, isopiscyderrone, lupinalbin B, as well as 5 different methylated derivatives of luteone and 3 different methylated derivatives of genistein."

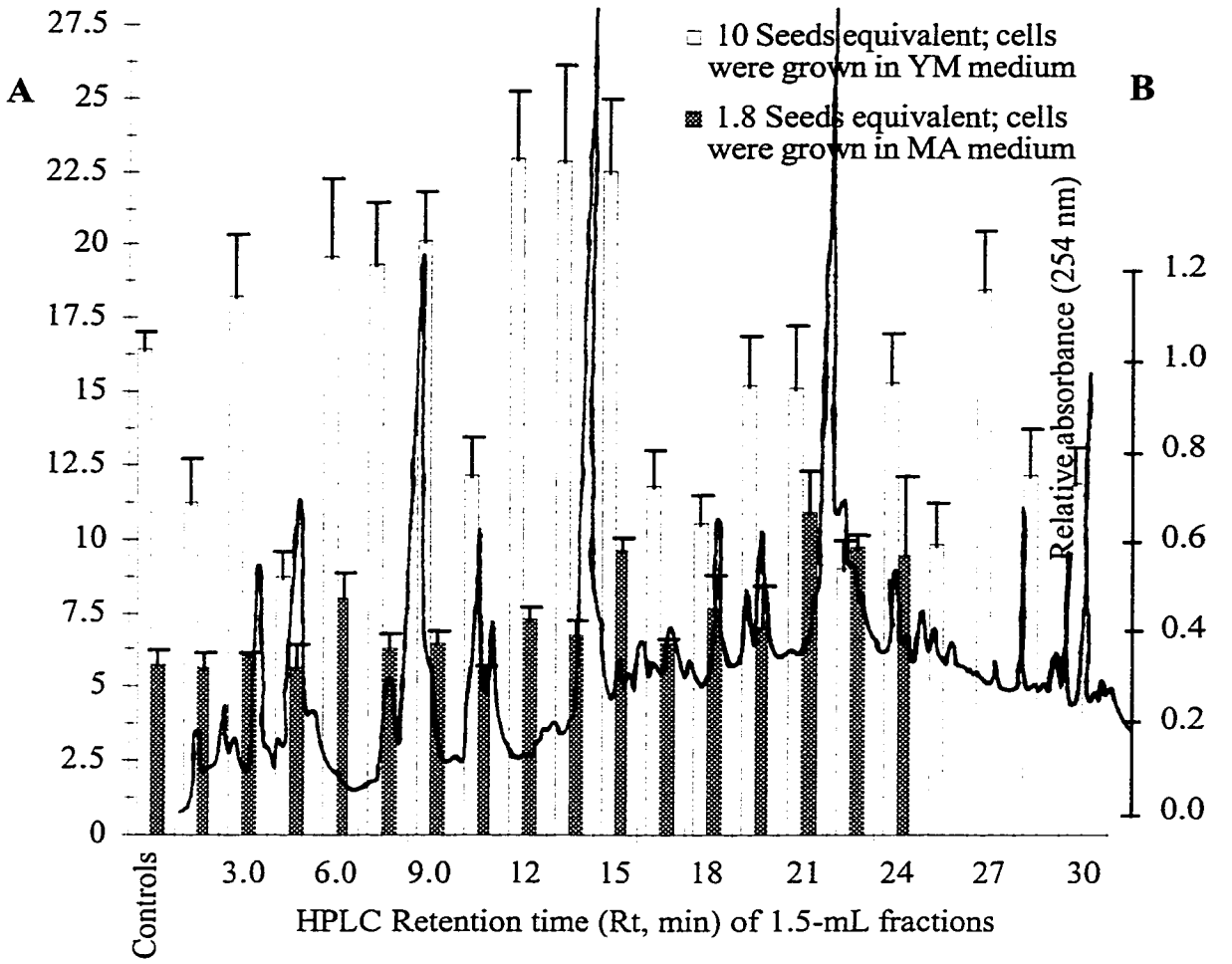


Figure 9. ß-Galactosidase activity of *R. lupini*/pRmM57 cultures treated with HPLC-fractionated root exudates of *L. albus*

(A) Relative ß-galactosidase activity in Miller units, and (B) relative absorbance at 254 nm of the fractionated root exudates. Root exudates from 3 to 7 d-old lupin seedlings were extracted, fractionated by gradient-HPLC (e.g. from 45% to 100% methanolic acetic acid/aq. acetic acid in 23 min; HOAc, 0.5% v/v). Twenty fractions of 1.5-min each were collected and used as induction mixtures for *R. lupini*/pRmM57 grown in YM or MA media. After a 20-h induction, duplicate ß-galactosidase assays were carried out. Non-fractionated root exudates gave 19.5 ±1.6 and 12.4±0.9 ß-galactosidase units after either 10 or 1.8 seed equivalent, respectively. Treatments were made equivalent to either 10 or 1.8 HPLC-fractionated seed equivalent. Standard errors are indicated by the bars above each plotted value.

Table 10. ß-Galactosidase activity of *R. lupini* /pRmM57 grown in YM medium after a 20 h induction by different concentrations of isoflavonoids[1]

Flavonoid tested	2.0 μM		4.0 μM		10 μM		20μM	
	M. units[2] (U)	SE[3] (%)	M. units (U)	SE (%)	M. units (U)	SE (%)	M. units (U)	SE (%)
Control[4]	13.6	6	13.6	6	13.6	6	13.6	6
Genistein-7-*O*-Glc	-9.2	12	13.2	*	16.1	14	7.7	23
2'-OHgenistein-7-*O*-Glc	22.8	6	13.1	11	15.7	11	6.6	18
2'-Hydroxygenistein	-7.1	*	12.3	12	14.6	30	5.6	16
Genistein	14.7	8	14.2	20	10.6	24	16.3	31
Lupinalbin A	5.6	9	13.6	19	13.0	*	10.0	30
Luteone	9.9	*	14.1	*	11.0	65	4.1	*
Licoisoflavone A	-6.9	6	14.3	*	16.8	*	6.86	8
Wighteone	10.0	7	11.4	12	8.4	17	8.2	13
Lupiwighteone	10.0	13	13.0	8	11.9	10	15.6	15
Derrone	17.2	6	33.5	6	14.7	*	4.5	40
2'-Hydroxylupalbigenin	6.8	8	4.3	11	12.5	12	4.2	37
Lupalbigenin	22.5	8	13.5	*	9.0	39	11.9	9

[1]Cultures of *R. lupini*/pRmM57 grown in YM medium were diluted to an OD_{600} of 0.1 and induced with the test isoflavonoids for a period of 20 h. Following induction, two aliquots of 100 μL from each culture were assayed for ß-galactosidase activity for a period of 4 h, and each assay was monitored at OD_{420} after centrifugation. Abbreviations: Glc, glucoside.

[2]Averages of duplicate ß-galactosidase assays.

[3]Standard error (% of SE) of averaged ß-galactosidase assays; *, SE<5%.

[4]Control cultures were treated with 80% aq. MeOH.

gave any significant result, except for the genistein monoprenyls, as well as lupalbigenin and derrone which resulted in very low, but detectable ß-galactosidase activities (data not shown). It should be noted that these *nodD1*⁻ *R. lupini*/pRmM57 transformants were resistant to spectinomycin, but not to tetracyclin, and they did not respond to luteolin induction.

E.4.4. Inducibility of *Nod* Genes in *R. lupini*/pRmM57 after treatment with Aldonic Acids

Molecules other than isoflavones, such as aldonic acids, were used in this study for the induction of *R. lupini*/pRmM57 transformant cultures. Since betaines (Figure 1A) have been reported to induce *R. meliloti nod* gene expression at a 10^3-fold higher concentration than that required for similar levels of induction with luteolin (Phillips *et al.* 1992), therefore, aldonic acids were used in this experiment at millimolar concentrations. Figure 10 shows the net ß-galactosidase activities after incubation with different concentrations of aldonic acids ranging from 100 µM to 80 mM. These results show that the relative ß-galactosidase activities actually increase in a dose-dependent manner with both erythronic and tetronic acids, but not with succinic acid, a structural analog of aldonic acids. Therefore, erythronic and tetronic acids may be considered as *nod* gene inducers in *R. lupini*.

In order to further investigate this finding, the possible synergistic effect between erythronic acid and lupin isoflavones was tested, using a combination of 20 mM erythronic acid and 5 µM each of a number of lupin isoflavones. The ß-galactosidase activities, reported in Table 11, show that of all the isoflavones tested, lupiwighteone in combination with 20 mM erythronic acid was found to promote the highest relative ß-galactosidase activity, as compared with the erythronic acid control. Furthermore, treatment of *R. lupini*/pRmM57 with different combinations of lupiwighteone (0.05 to 5 µM), erythronic acid (5 to 20 mM) and 10 mM tetronic acid, showed that the highest relative ß-galactosidase activities were observed with a mixture of 5 µM lupiwighteone with either 20

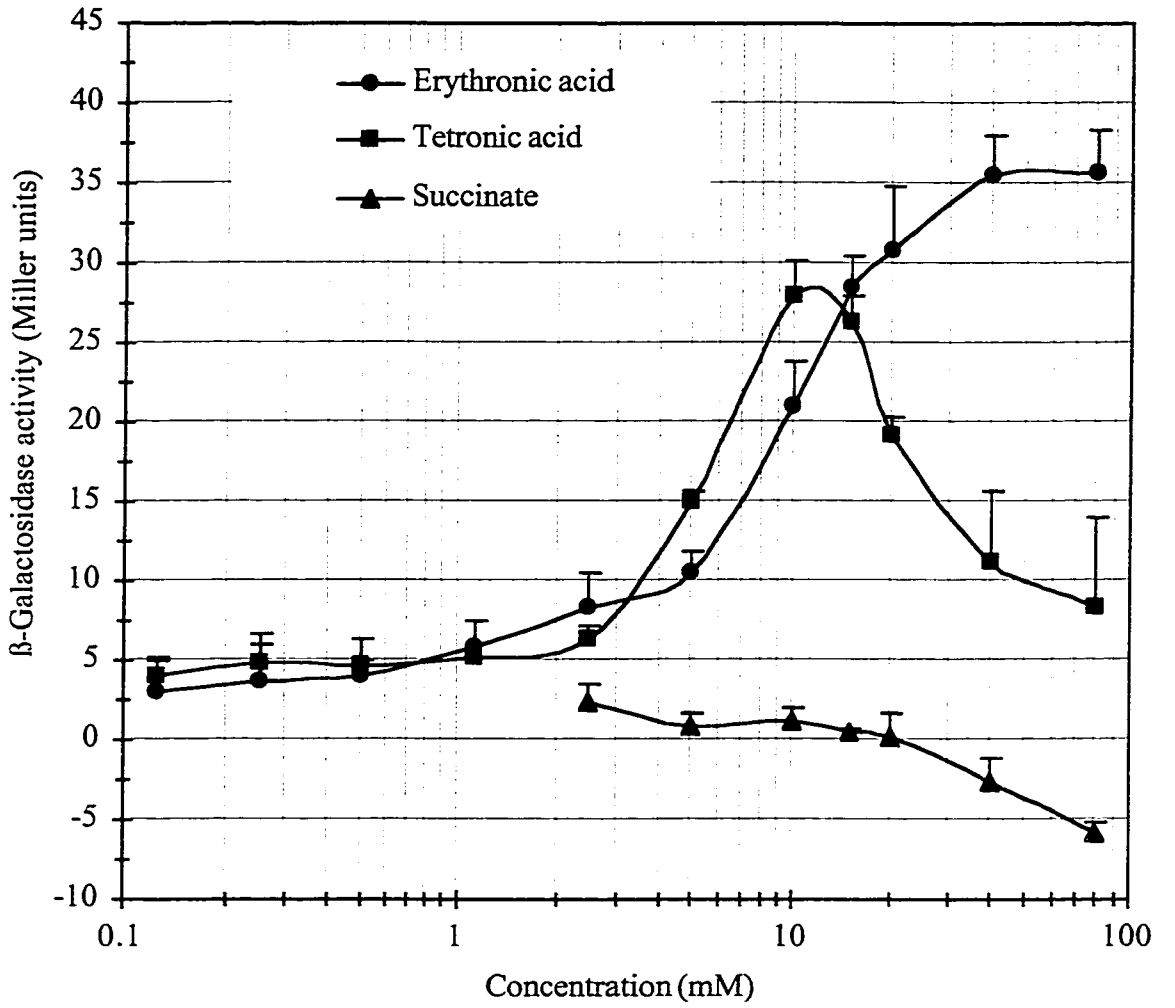


Figure 10. Effect of various aldonic acid concentrations on ß-galactosidase activity of *R. lupini*/pRmM57

Duplicate *R. lupini*/pRmM57 cultures, grown in YM medium, were induced for 20 h with micro- and millimolar concentrations of erythronic acid (●), tetronic acid (■), and succinic acid (▲) . Following induction, triplicate ß-galactosidase assays were carried on each culture. In addition, the ß-galactosidase activity of non-induced controls (i.e. treated with water) was monitored (ca. 18 U). The latter activity was subtracted from those obtained after aldonic acid treatments, to calculate the net values plotted above. Standard errors are indicated by the bars above each plotted value.

Table 11. ß-Galactosidase activity of *R. lupini*/pRmM57 grown in YM medium after a 20 h induction with 5 μM isoflavonoids and/or 20 mM erythronic acid[1]

Isoflavonoid tested (5 μM)	Erythronic acid (mM)	Miller units[2] (U)	SE[3] (%)
Control[4]	0	16.4	7
Control[5]	20	45.4	*
Genistein	20	24.1	6
2'-Hydroxygenistein	20	34.2	9
Lupinalbin A	20	26.6	7
Luteone	20	39.1	15
Licoisoflavone A	20	31.2	13
Wighteone	20	39.7	9
Isowighteone	20	34.4	13
Lupiwighteone	20	52.4	*
2'-Hydroxylupalbigenin	20	39.3	12
Lupalbigenin	20	42.6	11
Derrone	20	45.9	8

[1]Duplicate cultures of *R. lupini* /pRmM57 grown in YM medium were diluted to an OD_{600} of 0.1 and induced with the test isoflavonoid in presence or absence of erythronic acid for a period of 20 h. Following induction, three aliquots of 100 μL from each culture were assayed for ß-galactosidase activity for a period of ca. 1 h, and each assay was monitored at OD_{420} after centrifugation.

[2]Averages of six ß-galactosidase assays.

[3]Standard errors (% of SE) of averaged ß-galactosidase assays; *, SE<5%.

[4]Control cultures were treated with 40% aq. MeOH.

[5]Control cultures were treated with 80% aq. MeOH.

mM erythronic acid or 10 mM tetronic acid (Table 12). Therefore, these results indicate that a genistein monoprenyl, lupiwighteone exerts a synergistic effect with either of the aldonic acids in promoting ß-galactosidase activity, and hence of *nod* gene induction.

Finally, in order to verify that the observed inducibility by aldonic acids of *lacZ* fusions in *R. lupini* was not due to the presence of the heterologous *R. meliloti nodD1* in pRmM57, a dose-response induction by aldonic acids was established with a *R. meliloti* 1021/pRmM57 transformant. Table 13A shows that a dose-response relationship actually exists between the concentration of tetronic acid and the increase in relative ß-galactosidase activity, but not with either erythronic acid or succinic acid. In addition, in order to assess its functionality under the assay conditions used, a dose dependency between luteolin concentration and ß-galactosidase activity was established with *R. meliloti*/pRmM57 as a positive control (Table 13B).

Taken together, these results confirm that erythronic acid induces (2-3 folds) without heterologous bias the nodulation genes in *R. lupini*, whereas the observed *lacZ* expression by tetronic acid cannot be solely attributed to the endogenous *nodDs* of *R. lupini*. Furthermore, these results demonstrate that tetronic acid acts as an inducer of *nod* genes in *R. meliloti*.

E.5. RADIOLABELING AND ACCUMULATION OF NOD METABOLITES (LCOs)

E.5.1. Radiolabeling of *Nod* Metabolites and TLC Analysis

Preliminary incorporation experiments carried out on naringenin-induced (5μM) *R. loti*/p2112, with radiolabeled precursors resulted in label incorporation of ^{14}C-GlcN and ^{14}C-HOAc into two putative LCO bands, which had similar relative mobilities on TLC (Figure 11A). These radiolabeled bands were not observed in either of the respective, non-induced controls. Furthermore, the fact that additional, minor bands appear in both induced and non-induced treatments administered ^{14}C-HOAc may be attributed to some label incorporation into fatty acids, with either different chain lengths or with different

Table 12. ß-Galactosidase activity of *R. lupini* /pRmM57 grown in YM medium after a 20 h induction with different concentrations of lupiwighteone and aldonic acids[1]

Lupiwighteone concentration	Control[2,3]	SE[4]	**EA**[5] 5 mM	SE (%)	**EA** 10 mM	SE (%)	**EA** 20 mM	SE (%)	**TA** 10 mM	SE (%)
Control	15.7	*	34.7	7	42.4	8	46.8	*	48.7	9
0.05 µM	17.9	*	34.8	*	42.4	9	47.3	*	49.4	*
0.1 µM	14.4	8	36.1	*	44.2	3	48.3	*	48.2	*
0.5 µM	19.2	6	35.7	*	42.7	*	48.9	*	49.8	*
1 µM	14.2	6	34.1	6	40.0	11	50.2	7	47.1	7
5 µM	15.6	*	37.0	*	41.0	6	51.5	6	52.6	10

[1]Cultures of *R. lupini*/pRmM57 grown in YM medium were diluted to an OD_{600} of 0.1 and induced with lupiwighteone in presence or absence of aldonic acids for a period of 20 h. Following induction, three aliquots of 100 µL from each culture were assayed for ß-galactosidase activity for a period of ca. 1 h, and each assay was monitored at OD_{420} after centrifugation.

[2]Control cultures were treated with 40% aq. MeOH and water.

[3]Averages of three ß-galactosidase assays.

[4]Standard error (% of SE) of averaged ß-galactosidase assays; *, SE<5%.

[5]Abbreviations: EA, Erythronic acid; TA, Tetronic acid.

Table 13. ß-Galactosidase activity of *R. meliloti* 1021/pRmM57 grown in YM medium after a 4 h induction with various concentrations of either aldonic acids (A), or luteolin (B)[1]

A Aldonic acid concentration	**EA**[2]	SE (%)[3]	**TA**	SE (%)	**SA**	SE (%)
1.0 mM	-0.26	*	-0.37	*	-0.10	*
2.5 mM	-1.45	*	2.81	*	0.29	*
5.0 mM	0.3	*	10.81	*	1.27	9
10 mM	-0.49	*	80.01	*	0.21	*
15 mM	ND		112	12	ND	
20 mM	0.39	*	ND		-0.60	*
40 mM	0.24	7	ND		ND	

B Luteolin concentration	ß-gal activity	SE (%)
10 nM	326	*
50 nM	484	*
100 nM	583	*
500 nM	617	*
1 µM	643	*
5 µM	662	6
10 µM	590	9
20 µM	417	15

[1]Duplicate cultures of *R. meliloti* 1021/pRmM57 grown in YM medium were diluted to an OD_{600} of 0.1 and induced with aldonic acids (A) or luteolin (B) for a period of 4 h. Following induction, three aliquots of 100 µL from each culture were assayed for ß-galactosidase activity, and each assay was monitored at OD_{420} after centrifugation. Net values shown are averages of six ß-galactosidase assays. Control cultures were treated with water and 80% aq. MeOH in A and B, respectively. Background ß-galactosidase activity was 21.5 units.

[2]Abbreviations. EA, Erythronic acid; TA, Tetronic acid; SA, Succinic acid.

[3]Standard error (% of SE) of averaged ß-galactosidase assays; *, SE<5%.

Label fed	Inducer	Dpm loaded
HOAc (1.25μCi)	-	13,740
HOAc (1.25μCi)	+	45,520
GlcN (0.5μCi)	-	8,180
GlcN (0.5μCi)	+	1/5th=48,000

Label fed	Strain	Dpm loaded
GalN (0.5μCi)	*R. loti*	8,560
GlcN (0.5μCi)	*R. loti*	1/6th=38,000
GalN (0.5μCi)	*R. lupini*	11,240
GlcN (0.5μCi)	*R. lupini*	13,200

Label fed	Inducer	Dpm loaded
Positive control (*R. loti*)		20,000
GlcN (0.5μCi)	-	13,120
GlcN (0.5μCi)	+	17,980
GalN (0.5μCi)	-	6,420
GalN (0.5μCi)	+	5,800
AcGlcN (0.5μCi)	-	10,600
AcGlcN (0.5μCi)	+	14,800
AcGalN (0.5μCi)	-	2,400
AcGalN (0.5μCi)	+	1,820
ManN (0.5μCi)	-	8,660
ManN (0.5μCi)	+	15,740
HOAc (2.8μCi)	-	16,450
HOAc (2.8μCi)	+	26,800

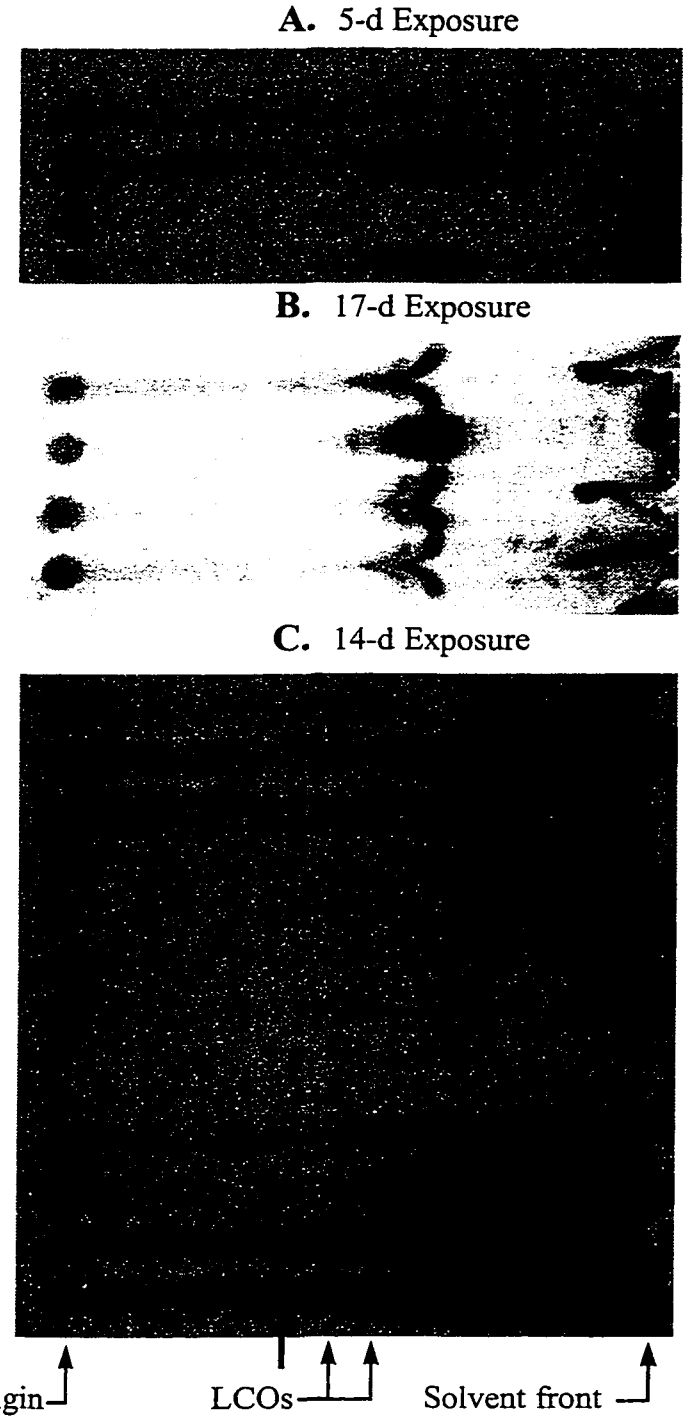


Figure 11. Metabolic radiolabeling of LCOs with various ^{14}C-labeled LCO precursors in *Rhizobium* strains harboring FITA constructs

Six-mL cultures (OD_{600} of 0.1) of p2112 transformants grown in YM medium were induced with 5 μM naringenin (+) or not (-), administered ^{14}C-labeled precursors and incubated for 21 h (A,B) or 40 h (C). *R. loti* and *R. lupini* transformants were used in A and C, respectively; whereas in B, each treatment included naringenin. Abbreviations: HOAc, acetic acid; Glc, glucose; N, amine; Gal, galactose; Man, mannose; Ac, acetyl.

degrees of unsaturation. As would be expected, acetate is a much less specific precursor of LCO biosynthesis, as compared to glucosamine (Figure 11A).

In order to optimize the relative yield of LCOs, various concentrations of naringenin were used (ca. 2.5, 5 and 10μM) prior to incorporation of ^{14}C-GlcN in *R. loti* /p2112. Of these, 5 μM naringenin resulted in the best relative LCO yield (ca. 32% of label fed), whereas the remainder was found in both the cell pellet and the extracted supernatant (52% and 16%, respectively). Furthermore, an induction period of 3 h was required prior to administration of the labeled precursor; since the omission of such a pre-incubation period reduced the relative LCO yield by 2-fold (data not shown). In contrast with ^{14}C-GlcN which gave the best relative LCO yield with *R. loti* /p2112, ^{14}C-HOAc resulted in only 4% product when used as a precursor (data not shown).

The putative radiolabeled LCO bands, obtained from naringenin-induced *R. loti*/p2112 were recovered from the TLC plates and analyzed by HPLC, in order to verify their identity (Figure 12). The relative activity profile obtained with a mixture of ^{14}C-HOAc and ^{14}C-GlcN shows that most of the radioactivity eluted at Rt 20.75-22.25 min, a region that corresponds to that of the LCO standards (see section E.5.4). In addition, enzyme hydrolysis and TLC analysis of the radiolabeled LCO bands recovered from the ^{14}C-HOAc and ^{14}C-GlcN feedings (Figure 13) show that both the unhydrolyzed control and the proteinase K hydrolysate exhibit similar TLC mobilities, which is not unexpected since LCOs are not proteins. Furthermore, the fact that hydrolysis with either *N*-acylase, chitinase, *N*-acetylglucosaminidase and egg white lysozyme gives additional radiolabeled bands, with relative TLC mobilities different from those of the control (Figure 13A and B), demonstrates that the hydrolyzed molecules are indeed LCOs. Since an apparent shift in the relative mobility was observed with the chitinase treatment (Figure 13A and B), enzyme hydrolysis was repeated with ^{14}C-GlcN-labeled LCOs (Figure 13 C). In fact, a more evident shift was observed with the chitinase treatment (Figure 13C) which confirmed that hydrolysis had actually occured.

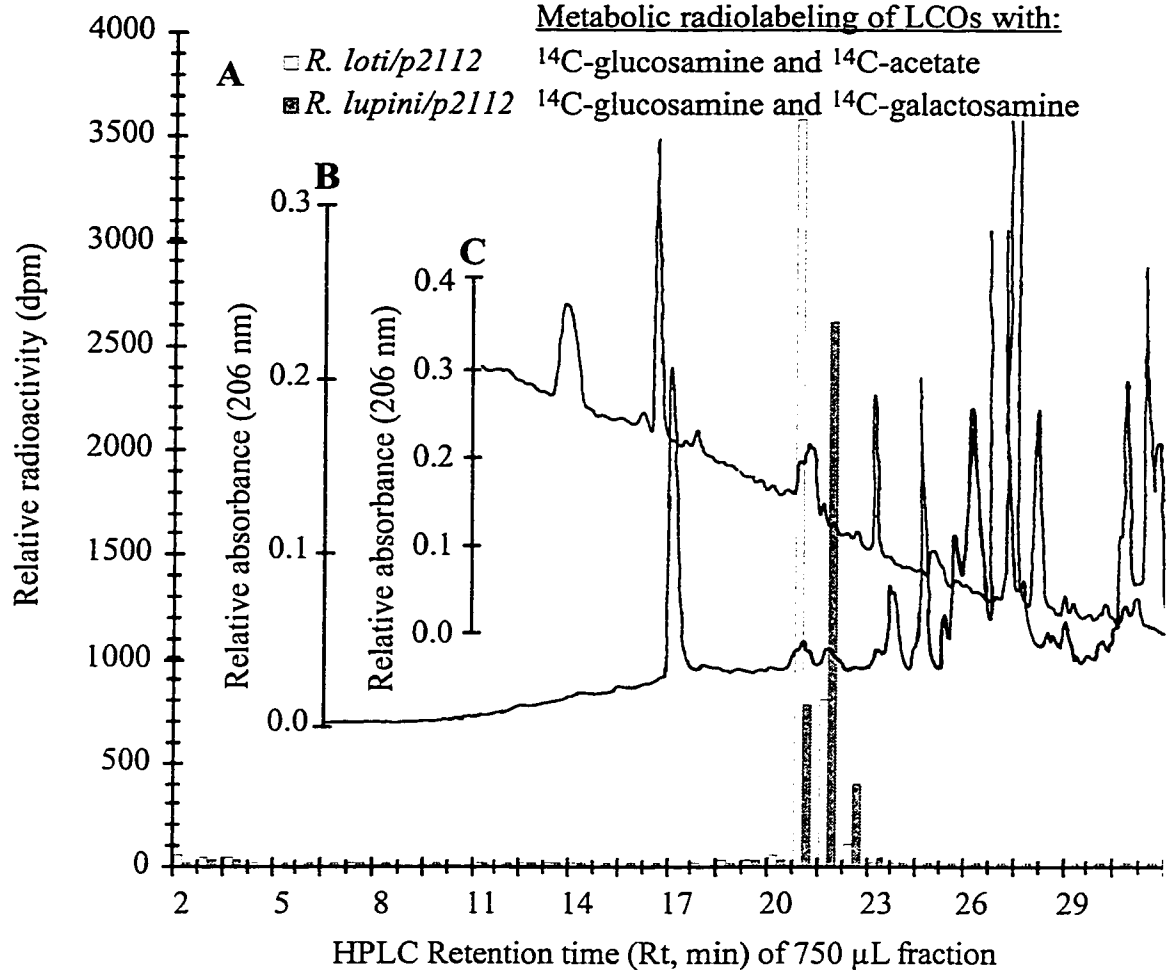


Figure 12. HPLC-fractionation of radiolabeled LCOs from *Rhizobium* strains harboring FITA constructs

(A) Relative activity of individual fractions; (B) and (C) relative absorbance at 206 nm after gradient HPLC-fractionation of LCOs. After induction with 5 µM naringenin for 3 h, *Rhizobium* cultures grown in YM medium were administered either ^{14}C-GlcN, ^{14}C-GalN or ^{14}C-HOAc for 18 h. After centrifugation, culture-supernatants were pre-purified on Sep-Pack, chromatographed on TLC with 50% aq. AcN and autoradiographed. The radiolabeled bands were recovered from autoradiographed TLCs shown in Figure 11A and B for *R. loti* and *R. lupini*, respectively, and chromatographed by gradient-HPLC (from 15% to 40% aq. AcN in 13 min, followed by an increase to 100% AcN in 10 min). Fractions were collected every 45 seconds, and each was monitored by liquid scintillation counting in order to correlate individual activities (A) with retention times and elution profiles of *R. loti* and *R. lupini* (B and C, respectively).

Enzyme utilized	Units used (U)
Control (unhydrolyzed)	none
Porcine acylase	250 U
Chitinase	0.1 U
N-Acetyl glucosaminidase	0.25 U
Egg white lysozyme	1.0 U
Proteinase K	0.2 U
Control (unhydrolyzed)	none
Porcine acylase	250 U
Chitinase	0.1 U
N-Acetyl glucosaminidase	0.25 U
Egg white lysozyme	1.0 U
Proteinase K	0.2 U
Control (undigested)	none
Egg white lysozyme	10 U
Porcine acylase	250 U
Chitinase	0.1 U
N-Acetyl glucosaminidase	0.25 U

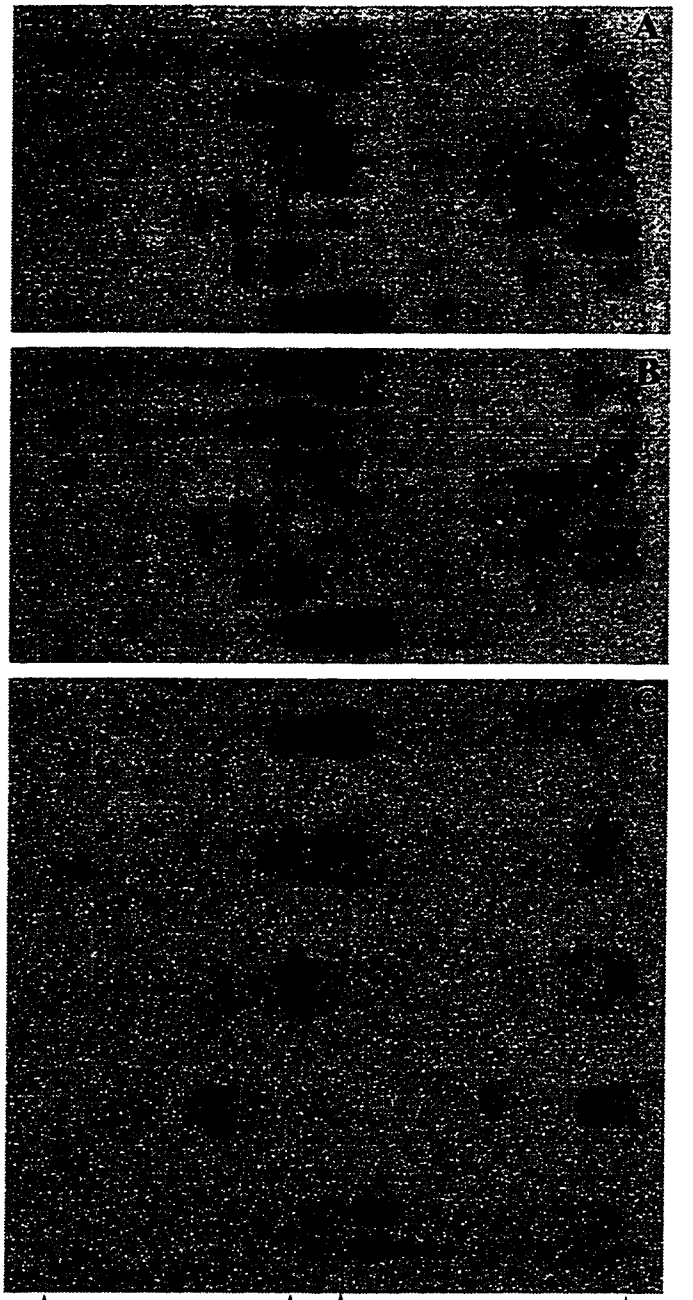


Figure 13. Hydrolysis of radiolabeled LCOs with various enzymes

The naringenin-induced (5.0 μM), radiolabeled LCOs of *R. loti*/p2112 grown in YM medium were recovered from TLCs according to autoradiograms of Figure 11A, and subjected to enzyme hydrolysis for 24 h at 30°C. The digestion patterns shown in panel A are from ^{14}C-HOAc incorporation, whereas those in panels B and C are from ^{14}C-GlcN incorporation. The chromatographed TLCs were autoradiographed for 20 d in A and B, and for 5 d in C.

The radiolabeled LCO bands of each of the chromatographed hydrolysates (Figure 13B) were recovered from the TLC plates and subjected to HPLC analysis in order to further establish their chemical identities. The relative enzyme-radioactivity pattern obtained from the 40 fractions collected (Figure 14), supports again the same conclusion; that is the elution-radioactivity pattern of the LCO-specific enzyme treatments are different from those of the control or proteinase K. These results further support the notion that radioactive bands produced by *R. loti*/p2112 (Figure 11A) are in fact LCOs, as indicated by their hydrolysis pattern after treatment with different enzymes. Therefore, the *R. loti*/p2112 LCOs were used as positive controls in all other experiments, since their identity has been verified, and because naringenin-induction is known to promote a very efficient LCO yield in FITA transformants of *R. loti*, as compared to that of *R. lupini* (Figure 11B).

Metabolic radiolabeling experiments were also carried out with FITA-harboring strains of naringenin-induced *R. lupini* and *R. loti*, in order to compare the relative efficiency of the two strains for the incorporation of different enantiomeric amino sugars. Figure 11B shows that the rate of ^{14}C-glucosamine incorporation in *R. loti*/p2112 is ca. 27-fold higher than that of ^{14}C-galactosamine. Whereas the transformed *R. lupini*/p2112 incorporated both labeled precursors to a similar extent, galactosamine seems to serve as a better precursor of LCOs than glucosamine, but with a much lower product yield. This is in contrast with *R. loti*/p2112, when administered ^{14}C-GlcN (Figure 11B). HPLC analysis of the ^{14}C-GlcN and ^{14}C-GalN labeled-LCO bands produced by *R. lupini*/p2112 gave an elution pattern with relative activities similar to that of the FITA-harboring *R. loti*. This is consistent with their being LCOs (Figure 12).

In order to determine which of the radiolabeled compounds is a better precursor of *R. lupini*/p2112 LCOs, equivalent amounts (0.5 μCi) of radioactivity were administered, except for HOAc which was supplied at 2.8 μCi. In addition, the incorporation period was extended to 40 h in order to allow a higher incorporation of radiolabel into LCOs. The results obtained (Figure 11C) show that glucosamine, and, to a lesser extent, its acetylated

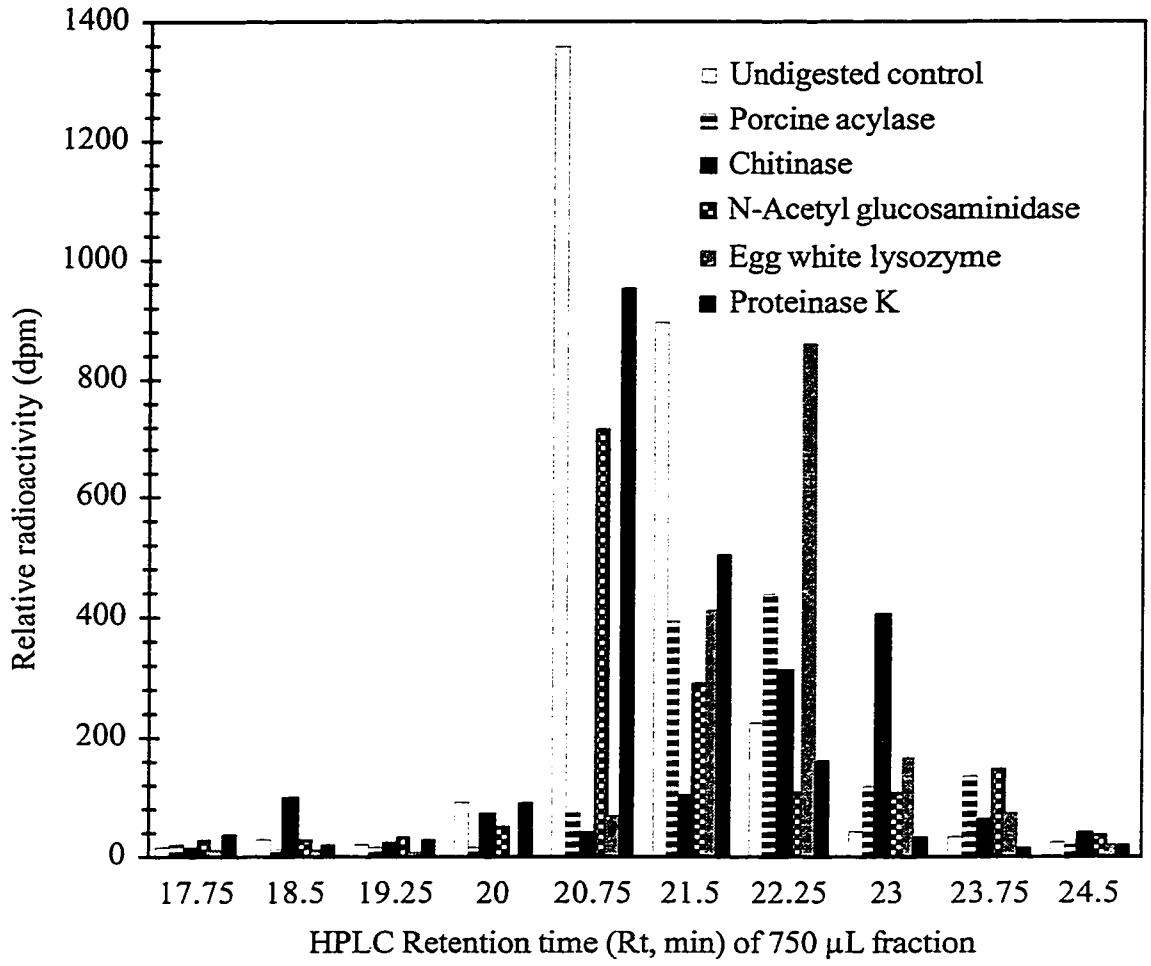


Figure 14. HPLC analysis of radiolabeled LCOs from *R. loti*/p2112 after hydrolysis with various enzymes

Relative activity (dpm) after gradient-HPLC fractionation (from 15% to 40% aq. AcN in 13 min, followed by an increase to 100% AcN in 10 min) of hydrolyzed ^{14}C-GlcN LCOs from *R. loti*/p2112. Fractions were recovered from TLC after autoradiogram shown in Figure 13B. For each of the hydrolysates recovered, 40-750 µL fractions were collected and scintillation counted.

derivative were the best LCO precursors, as compared with the other radiolabeled compounds used (^{14}C-GalN, ^{14}C-AcGalN and ^{14}C-ManN; Figure 11C), although, an additional band of an apparently lower polarity was observed in the case of ^{14}C-HOAc feeding (Figure 11C; bold arrow). However, these incorporation studies did not reveal which is the best precursor of LCOs in transformant *R. lupini*/p2112, mainly because of low incorporation of radioactivity.

E.5.2. Metabolic Radiolabeling of LCOs in Wild-Type *Rhizobium lupini*

E.5.2.1. Preliminary Experiments and Technical Considerations

Preliminary incorporation studies using *R. lupini* treated individually with 5 μM each of 11 different lupin isoflavones (shown in Table 11), or in combination with 2.5 mM tetronic or erythronic acids, were carried out using 0.5 μCi of ^{14}C-HOAc, ^{14}C-GlcN or ^{14}C-GalN as LCO precursors. Since liquid-liquid extractions of the treated cultures with *n*-BuOH resulted in a poor recovery of labeled LCOs, an improved extraction method was used, which utilized Sep-Pack pre-purification of the culture supernatants (D.6.1; Laeremans *et al.* 1996). None of these 72 different radiolabeling treatments resulted, after autoradiography, in any LCO incorporation, as compared with the positive control (data not shown).

Another attempt using a higher amount of label (2.8 μCi ^{14}C-HOAc) in the presence of various concentrations of aldonic acids, and for a longer incorporation period (40 h), met with little success (data not shown). It seems that the long incorporation period may have resulted in the production of excessive amounts of exopolysccharides in the cell cultures, which rendered them too viscous to allow for a good LCO yield .

Further attempts to enhance the extraction of LCOs, involved loading the centrifuged supernatants of naringenin-induced *R. loti*/p2112 cultures on the Sep-Pack filters, and elution with increasing concentrations of aqueous MeOH (20%, 40%, 60%, 80% and 100%) instead of using absolute MeOH . The results of these attempts indicate that the

majority of radiolabeled LCOs were eluted from the filters with 80% and 100% MeOH, whereas only 4% was eluted with 60% MeOH (Figure 15). In addition, this eliminates radiolabeled compounds of high polarity that migrate at the solvent front (i.e. those eluting with 20% and 40% MeOH; Figure 15). Therefore, it became evident that after washing the loaded Sep-Packs with 50% aq. MeOH, elution with absolute MeOH yields LCOs of a higher purity. Furthermore, the use of a specific precursor, such as ^{14}C-GlcN, instead of ^{14}C-HOAc, should result in a higher specific activity of radiolabeled LCOs.

E.5.5.2. LCO biosynthesis in Wild-Type *Rhizobium lupini*

The metabolic radiolabeling of LCOs was carried out with aldonic acid-treated *R. lupini* cultures using 2 μCi of ^{14}C-GlcN. The results (Figure 16) show that the radiolabeled LCOs, that exhibited relative mobilities similar to those of the positive control, decrease with increasing aldonic acid concentration. However, additional radiolabeled bands with a lower mobility on C18 silica, were also observed (Figure 16). The latter seem to increase with increasing concentrations of either erythronic acid or tetronic acid, but not with succinic acid. Therefore, it can be concluded that both aldonic acids promote LCO biosynthesis in wild-type *R. lupini*

Another incorporation study, using the optimized conditions (E.5.2.1) and 2 μCi of ^{14}C-GlcN, was carried out with a combination of 5 μM of either genistein, lupiwighteone or derrone, and aldonic acids (20 mM erythronic, 10 mM tetronic or 20 mM succinic acid). The results (Figure 17) show that the combined inductions with lupiwighteone and erythronic acid, or with derrone and erythronic/succinic acids result in a higher label incorporation into the putative LCO bands (Figure 17). Therefore, the combined treatments with both prenylated isoflavones and erythronic acid, or with derrone and succinate seem to exert a synergistic effect on LCO biosynthesis in wild-type *R. lupini* (Figure 17).

HPLC analysis of an aliquot of the putative LCO bands, recovered from the TLC plates (Figures 16 and 17), shows that most of the radioactivity was eluted at Rt 22.5-23.75 min

Percent MeOH (%)	Dpm loaded
20	23,200
40	8,950
60	9,700
80	151,800
100	80,950
LCO extract (partial sum)	274,600
Extracted medium	148,656
Cell pellet	246,420
Sum	669,670

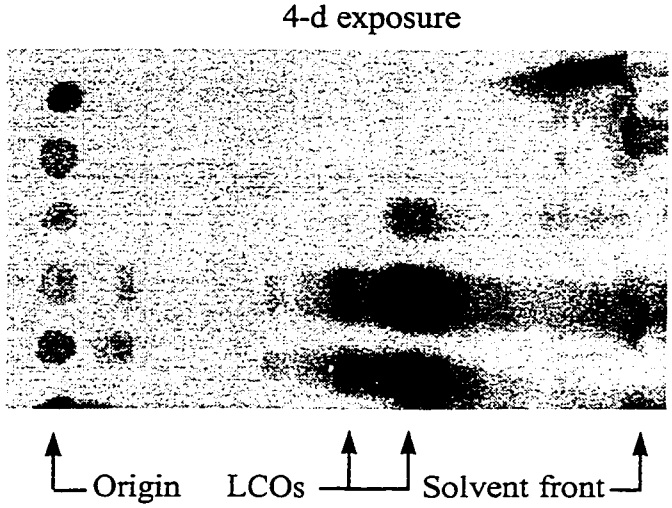


Figure 15. Sep-Pack elution of LCOs using different MeOH concentrations

The LCOs from naringenin-induced *R. loti*/p2112 were eluted with increasing concentrations of aq. MeOH (20%, 40%, 60%, 80% and 100%), after TLC analysis. Total radioactivity for the eluted LCOs, the extracted medium, as well as the cell pellet, are shown.

Aldonic acid tested		Dpm loaded
Positive control*		30,000
Negative control		4,460
EA	2.5 mM	4,820
EA	10 mM	3,860
EA	20 mM	6,060
EA	40 mM	6,200
TA	2.5 mM	4,020
TA	5 mM	5,200
TA	10 mM	4,900
TA	15 mM	3,960
SA	2.5 mM	4,680
SA	10 mM	7,700
SA	20 mM	8,180

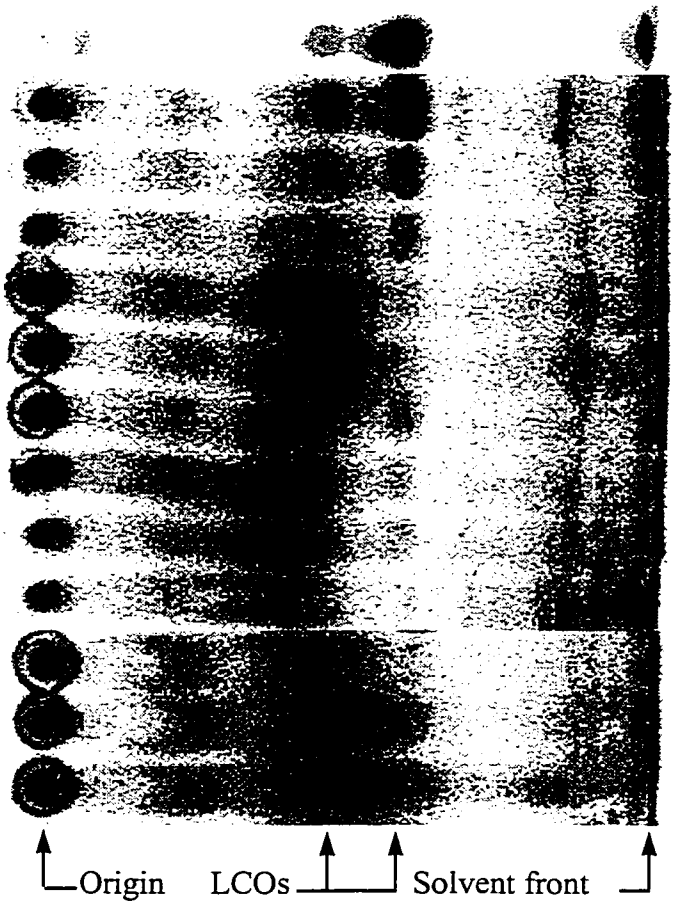


Figure 16. Metabolic radiolabeling of LCOs in *R. lupini* fed ^{14}C-glucosamine after treatment with various concentrations of aldonic acids

Six-mL cultures (OD_{600} of 0.1) of *R. lupini* grown in YM medium were induced for 3 h with various concentrations of aldonic acids and administered 2.0 μCi of ^{14}C-GlcN, for 18 h. After centrifugation, the supernatants were pre-purified on silica C18, washed with 50% aq. MeOH, eluted with MeOH, analyzed by TLC with 50% aq. AcN and autoradiographed for 5 d in a phosphoimager.

An aliquot (1/20) of each LCO-extract was scintillation counted and is shown opposite its "origin" as dpm loaded. Arrows indicate the relative migration of LCOs, as well as the origin and solvent front on the TLC autoradiogram.

*The positive control was obtained from 5 μM naringenin-treated *R. loti*/p2112 cultures.

Abbreviations: TA, tetronic acid; EA, erythronic acid; SA, succinic acid.

Compound(s) tested	Dpm loaded
Positive control*	30,000
Negative control	4,460
Genistein 5μM	5,600
Genistein +EA 20 mM	7,340
Genistein +TA 10 mM	6,740
Genistein +SA 20 mM	8,840
Lupiwighteone 5μM	5,960
Lupiwighteone +EA 20 mM	6,860
Lupiwighteone +TA 10 mM	3,740
Lupiwighteone +SA 20 mM	6,660
Derrone 5μM	5,860
Derrone +EA 20 mM	6,880
Derrone +TA 10 mM	5,420
Derrone +SA 20 mM	8,820

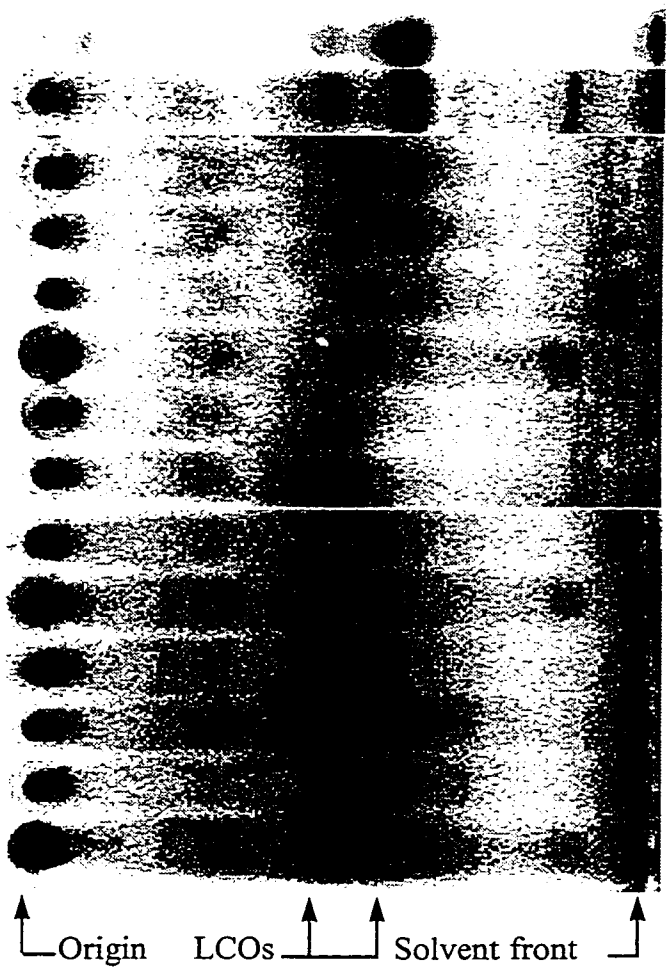


Figure 17. Metabolic radiolabeling of LCOs in *R. lupini* fed ^{14}C-glucosamine after treatment with a combination of aldonic acids and/or isoflavone

Six-mL cultures (OD_{600} of 0.1) of *R. lupini* grown in YM medium were induced for 3 h with a combination of isoflavones (5 μM) and/or aldonic acids and administered 2.0 μCi of ^{14}C-GlcN, for 18 h. After centrifugation, the supernatants were pre-purified on silica C18, washed with 50% aq. MeOH, eluted with MeOH, analyzed by TLC with 50% aq. AcN and autoradiographed for 5 d in a phosphoimager.

An aliquot (1/20) of each LCO-extract was scintillation counted and is shown opposite its "origin" as dpm loaded. Arrows indicate the relative migration of LCOs, as well as the origin and solvent front on the TLC autoradiogram.

*The positive control was obtained from 5 μM naringenin-treated *R. loti*/p2112 cultures.

(Figure 18); a retention time that is slightly delayed (ca. 1 to 2 fractions), as compared to that of the *R. loti*/p2112 LCOs (Figure 12). This elution behavior further suggests that these radiolabeled bands are less polar than those of *R. loti*. Enzyme hydrolysis of the remainder of the LCO bands, followed by HPLC analysis, gave an elution-radioactivity pattern that is different from both the control and proteinase K (Figure 19), which suggests that the hydrolyzed molecules are indeed LCOs.

E.5.3. Metabolic Radiolabeling of LCOs in Other Wild Type *Rhizobium Spp.*

Since aldonic acids were found to promote the metabolic radiolabeling of LCOs in *R. lupini*, the possibility that this phenomenon takes place in *R. loti* was investigated, in spite the fact that the signal molecule(s) in this species remains to be determined. Radiolabeling of LCOs was carried out using 0.5 μCi of ^{14}C-GlcN and *R. loti* cultures that were induced with different concentrations of aldonic acids. The results (Figure 20) show that, as compared to the positive control, the production of radiolabeled LCOs increased with increasing concentrations of tetronic acid, but not with either erythronic or succinic acids. Therefore, tetronic acid seems to act as a *nod* gene inducer in wild-type *R. loti*, since a dose dependency exists between tetronic acid and LCO biosynthesis. In addition, treatment of *R. loti* with a 20 mM succinate seems to promote such an effect in this bacterial species. However, this result should be interpreted with caution due to the fact that succinate is a common metabolite that may not be involved in the induction process.

Since tetronic acid was found to induce *lacZ* expression, and hence, *nod* gene induction in *R. meliloti*/pRmM57 (Table 13), LCO biosynthesis using 0.5 μCi ^{14}C-GlcN was carried out in wild-type *R. meliloti* after induction with either 20 mM erythronic acid, 10 mM tetronic acid, or with 5 μM luteolin, the natural flavonoid inducer. The results (Figure 21A) show that radiolabeled LCOs were produced after induction with tetronic acid, but not with erythronic acid, based on the relative mobilities of the radiolabeled spots that were similar to those of the luteolin-induced positive control (Figure 21A). It is interesting to

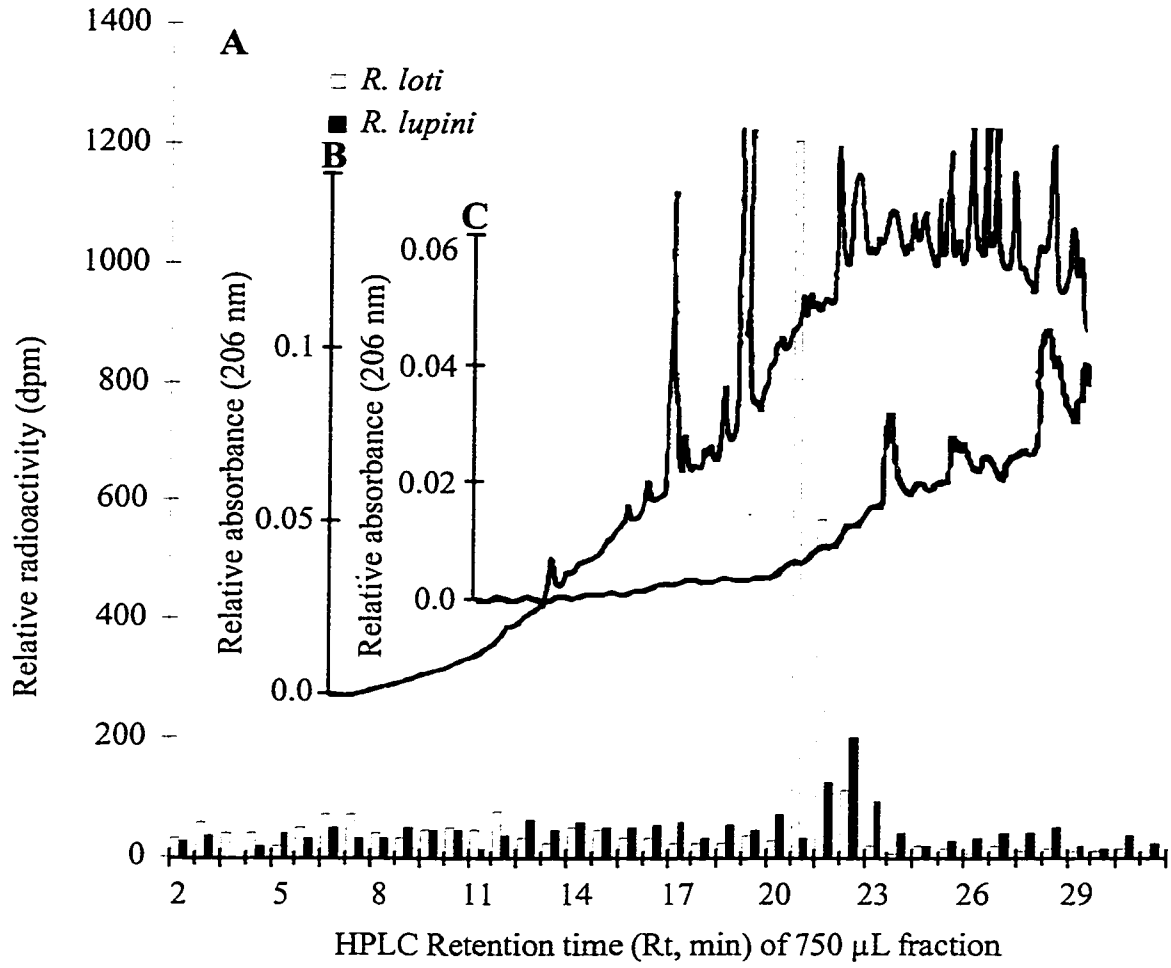


Figure 18. HPLC-fractionation of radiolabeled LCOs from wild-type *Rhizobium* strains

(A) Relative activity of individual fractions; and (B) and (C) Relative absorbance at 206 nm after gradient HPLC-fractionation of radiolabeled LCOs. After 3 h induction with aldonic acids, *Rhizobium* cultures grown in YM medium were administered ^{14}C-GlcN for 18 h. After centrifugation, culture-supernatants were pre-purified on Sep-Pack, chromatographed on TLC with 50% aq. AcN and autoradiographed. The radiolabeled bands were recovered from autoradiographed TLC shown in Figures 16-17 and 20 for *R. lupini* and *R. loti*, respectively, and chromatographed by gradient-HPLC (from 15% to 40% aq. AcN in 13 min, followed by an increase to 100% AcN in 10 min). Fractions were collected every 45 seconds, and each was monitored by scintillation counting in order to correlate individual activities (A) with retention times and elution profiles of *R. loti* and *R. lupini* (B and C, respectively).

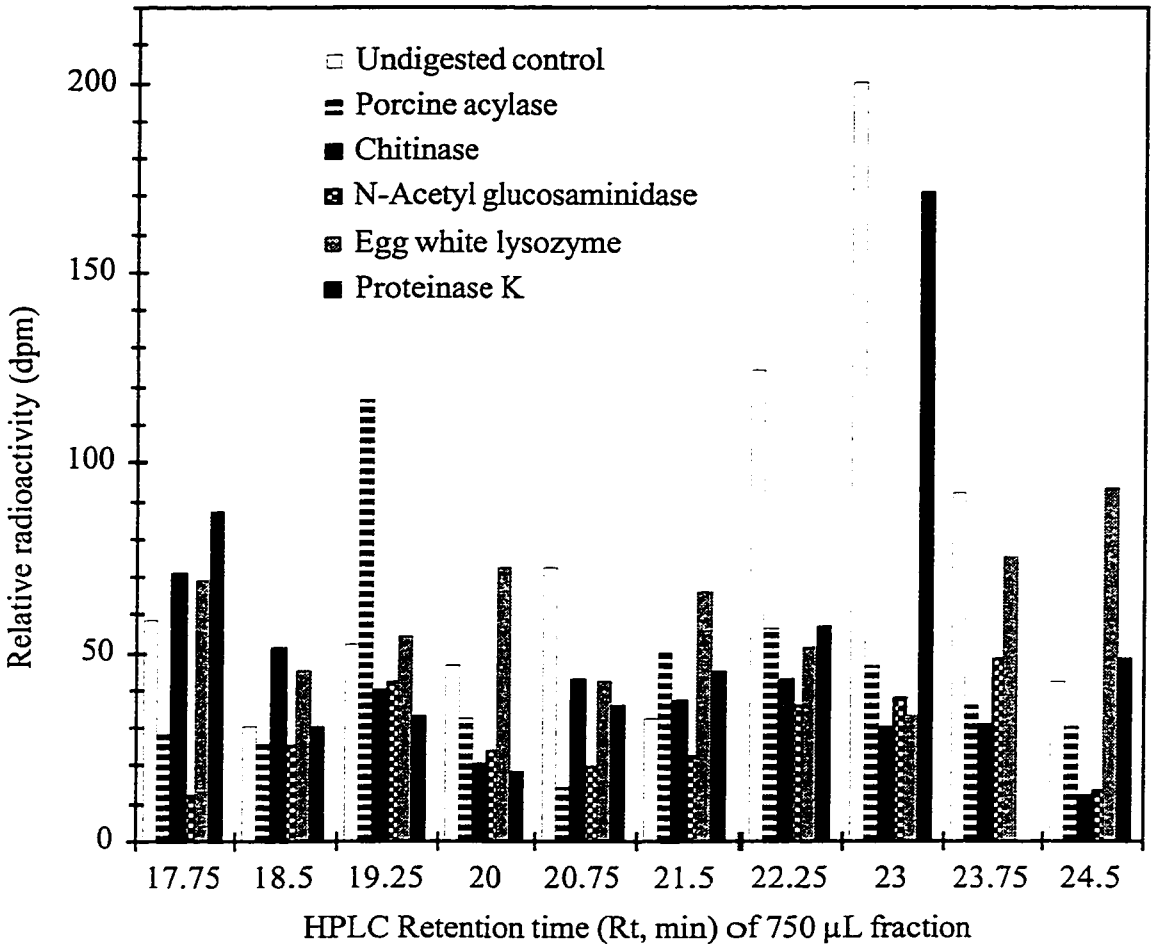


Figure 19. HPLC analysis of *R. lupini* radiolabeled LCOs after hydrolysis with various enzymes

Relative activity (dpm) after gradient-HPLC fractionation (from 15% to 40% aq. AcN in 13 min, followed by an increase to 100% AcN in 10 min) of hydrolyzed ^{14}C-GlcN LCOs from *R. lupini*. LCOs were recovered from autoradiographed TLCs shown in Figures 16 and 17. For each of the hydrolysates recovered, 40-750 µL fractions were collected and scintillation counted.

Inducer	Conc.	Dpm loaded
Positive control*		36,000
TA	2.5 mM	10,040
TA	5 mM	9,260
TA	10 mM	13,060
TA	15 mM	32,040
SA	2.5 mM	11,340
SA	5 mM	9,100
SA	10 mM	9,620
SA	20 mM	10,920
Positive control		36,000
Negative control**		9,440
EA	2.5 mM	7,860
EA	5 mM	9,360
EA	10 mM	8,040
EA	20 mM	7,400
EA	40 mM	9,160

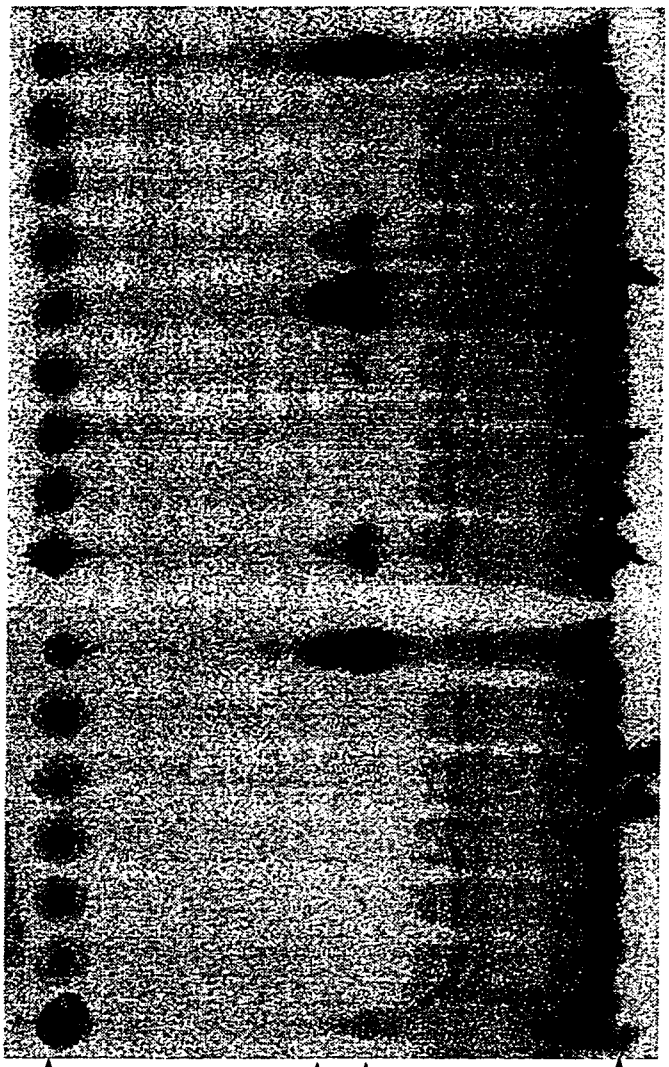


Figure 20. Metabolic radiolabeling of LCOs in *R. loti* fed ^{14}C-glucosamine after treatment with various concentrations of aldonic acids

Six-mL cultures (OD_{600} of 0.1) of *R. loti* grown in YM medium were induced for 3 h with various concentrations of aldonic acids and administered 0.5 µCi of ^{14}C-GlcN, for 18 h.LCO biosynthesis in wild-type *R. loti* after induction with aldonic acids. After centrifugation, the culture-supernatants werepre-purified on silica C18, analyzed by TLC with 50% aq. AcN and autoradiographed for 17 d.

Abbreviations: TA, tetronic acid; SA, succinic acid; EA, erythronic acid.

*The positive control was obtained from 5 µM naringenin-induced *R. loti/p2112*, equivalent to that of the highest treatment of the wild type (ca. 15 mM TA). **The negative control consisted of wild-type *R. loti* treated with water.

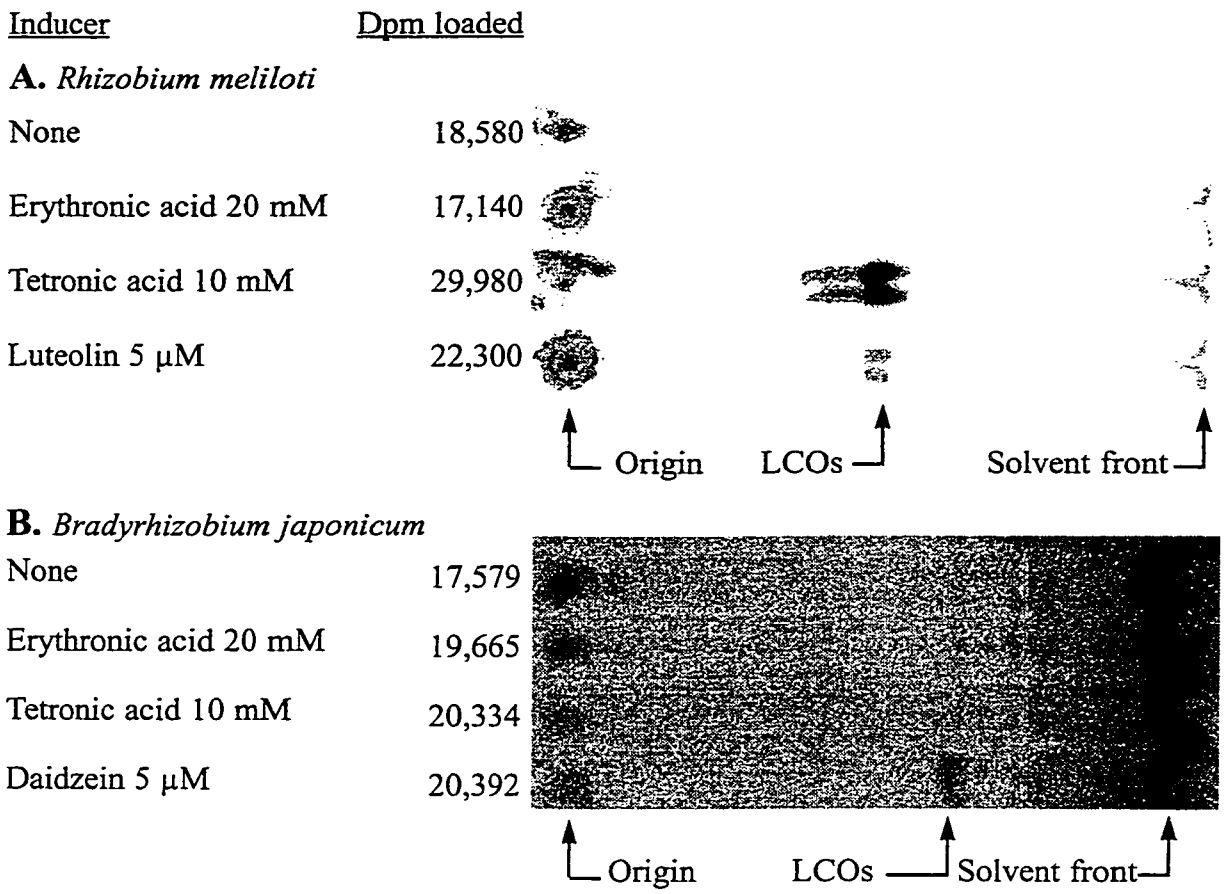


Figure 21. Metabolic radiolabeling of LCOs in wild-type *R. meliloti* and *B. japonicum* fed ^{14}C-glacosamine after induction with various aldonic acids or inducers

Six-mL cultures of *Rhizobium* grown YM medium(OD_{600} of 0.1) were induced for 3 h with aldonic acids or flavonoid inducers, administered 0.5 µCi of ^{14}C GlcN 18 h (A,B) or 42 h (C). After centrifugation, the supernatants were pre-purified on silica C18, prior to loading onto TLC plates. The latter were developed three times with aq. 80% MeOH (A) or 50% aq. AcN (B) and autoradiographed for 5 d (A, phosphoimager) or 17 d (B).

note that induction with 10 mM tetronic acid seems to promote a higher rate of incorporation into LCOs, as compared to that of luteolin; in spite of the fact that a 2000-fold higher concentration of tetronic acid was used. This result contrasts with those obtained with *lacZ* fusions (Table 13) where induction with tetronic acid resulted in an 8-fold lower ß-galactosidase units than with luteolin at similar concentrations. In order to verify the identity of the radiolabeled bands from induced *R. meliloti,* the putative LCO bands were recovered from the TLC plates (Figures 21A), and aliquots (ca. 2800 dpms each) were subjected to enzyme hydrolysis and HPLC analysis. The relative enzyme-radioactivity pattern obtained (Figure 22) indicates that LCO-specific enzyme treatments are different from those of both the control and proteinase K, and supports the notion that the hydrolyzed molecules are LCOs. These results strongly suggest that tetronic acid promotes LCO biosynthesis in *R. meliloti.*

Similar experiments carried out with *B. japonicum, R. leg.* bv *trifolii* and *R. leg.* bv *viciae* (Figure 21B, 23A and 23 C, respectively) resulted in the metabolic radiolabeling of LCOs after induction with their respective inducers: daidzein, 7-4', dihydroxyflavone and apigenin, but not with either of the aldonic acids tested. Therefore, aldonic acids do not promote LCO biosynthesis in these *Rhizobium spp.*

E.5.4. LCO Accumulation *in vitro*

The *in vitro* accumulation of LCOs was carried out with larger volumes (ca. 30 mL) of induced *Rhizobium* cultures, in order to allow for the spectral analysis after HPLC elution, and for the purification of LCOs to be used for root hair deformation assays on the host, *L. albus.* Preliminary gradient HPLC analysis was achieved using a LCO mixture, derived from induced *R. loti* and *R. leg.* bv *viciae* generously supplied by Dr. H. P. Spaink (Leiden University, The Netherlands). Figure 24A shows that most of the LCOs eluted between Rt 20.7 and 22.5 min. LCOs in *R. loti*/p2112 induced with 5 µM naringenin exhibited a multi-apex peak with similar retention time that coincided with those

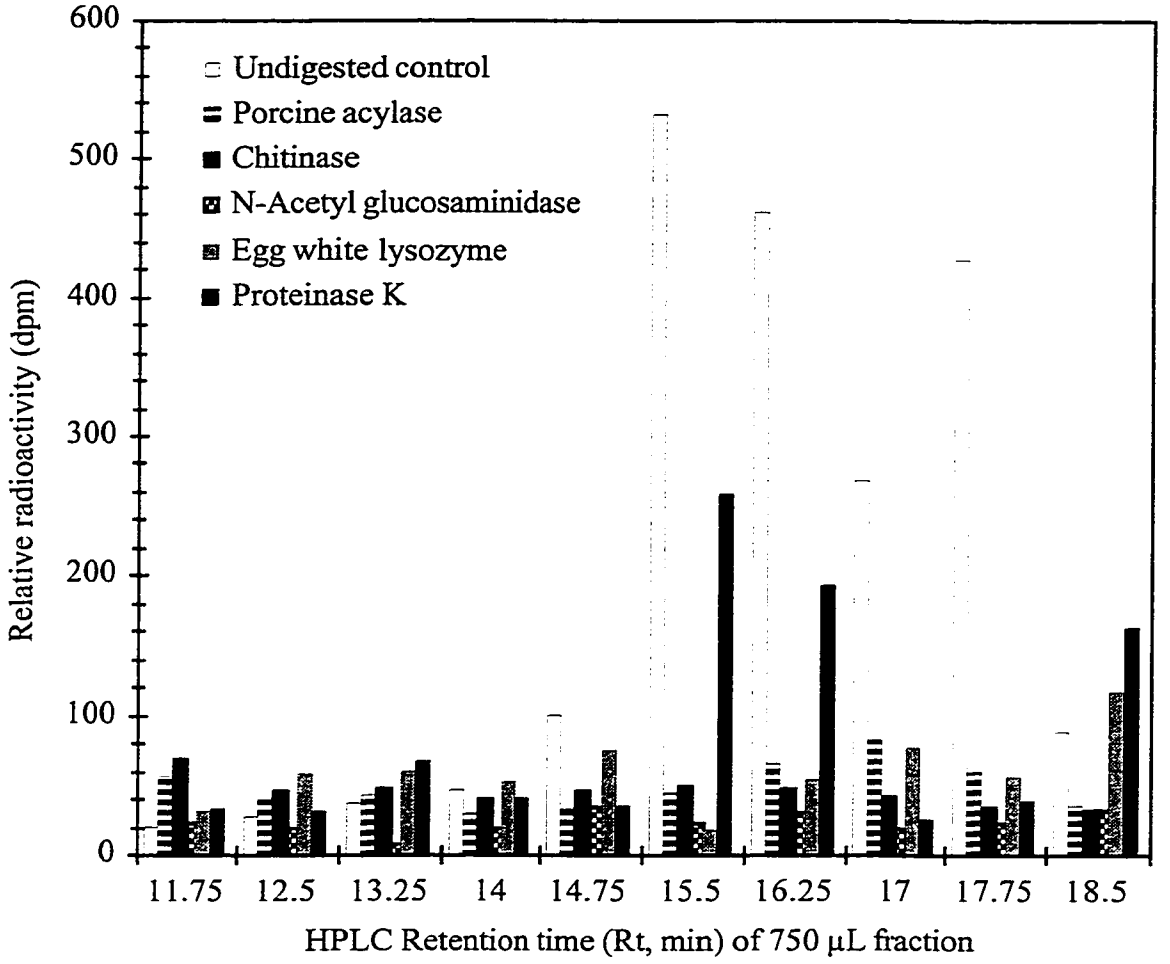


Figure 22. HPLC analysis of radiolabeled LCOs from wild-type *R. meliloti* after hydrolysis with various enzymes

Relative activity (dpm) after gradient-HPLC fractionation (from 15% to 40% aq. AcN in 13 min, followed by an increase to 100% AcN in 10 min) of hydrolyzed ^{14}C-GlcN labeled LCOs from wild-type *R. meliloti.* LCOs were recovered from TLC after autoradiography shown in Figure 21A. For each of the hydrolysates recovered, 40-750 µL fractions were collected and scintillation counted.

Inducer	Dpm loaded
A. *Rhizobium leg.* bv *trifolii*	
7-4',DiOH flavone 5 µM	49,120
None	20,940
Erythronic acid 20 mM	18,160
Tetronic acid 10 mM	17,960
Succinic acid 20 mM	15,600
B. *Rhizobium leg.* bv *viciae*	
Apigenin 5 µM	19,240
None	16,640
Erythronic acid 20 mM	13,360
Tetronic acid 10 mM	17,480
Succinic acid 20 mM	4,660

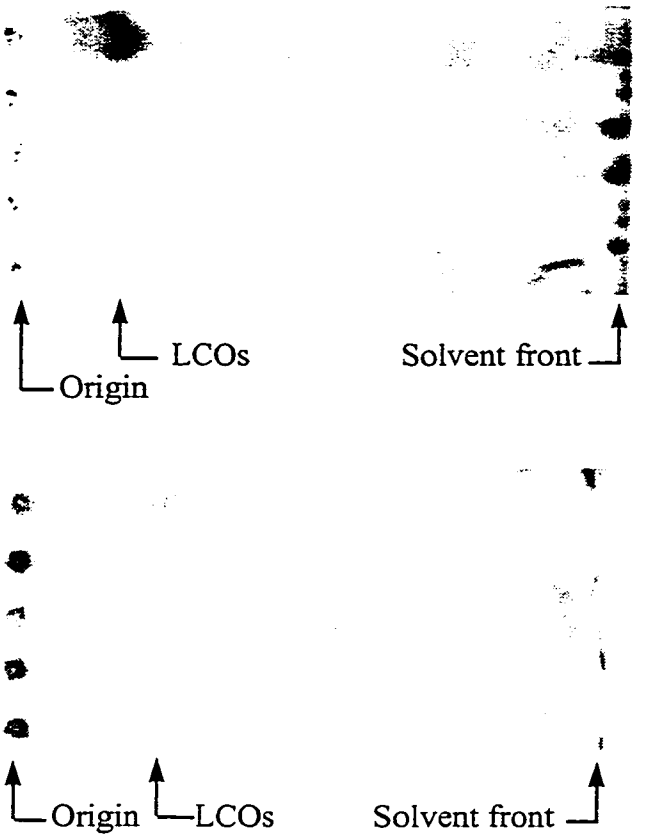


Figure 23. Metabolic radiolabeling of LCOs in wild-type *R. leg.* bv *trifolii* and *viciae* fed ^{14}C-glucosamine after induction with various aldonic acids or inducers

Six-mL cultures of *Rhizobium* grown GM medium (OD_{600} of 0.1) were induced for 3 h with various inducers, and administered 0.5 µCi of ^{14}C-GlcN 24 h (A) or 28 h (C). After centrifugation, the supernatants were pre-purified on silica C18, prior to loading onto TLC plates. The latter were developed, three times with 50% aq. AcN, and autoradiographed for 5 d in a phosphoimager.

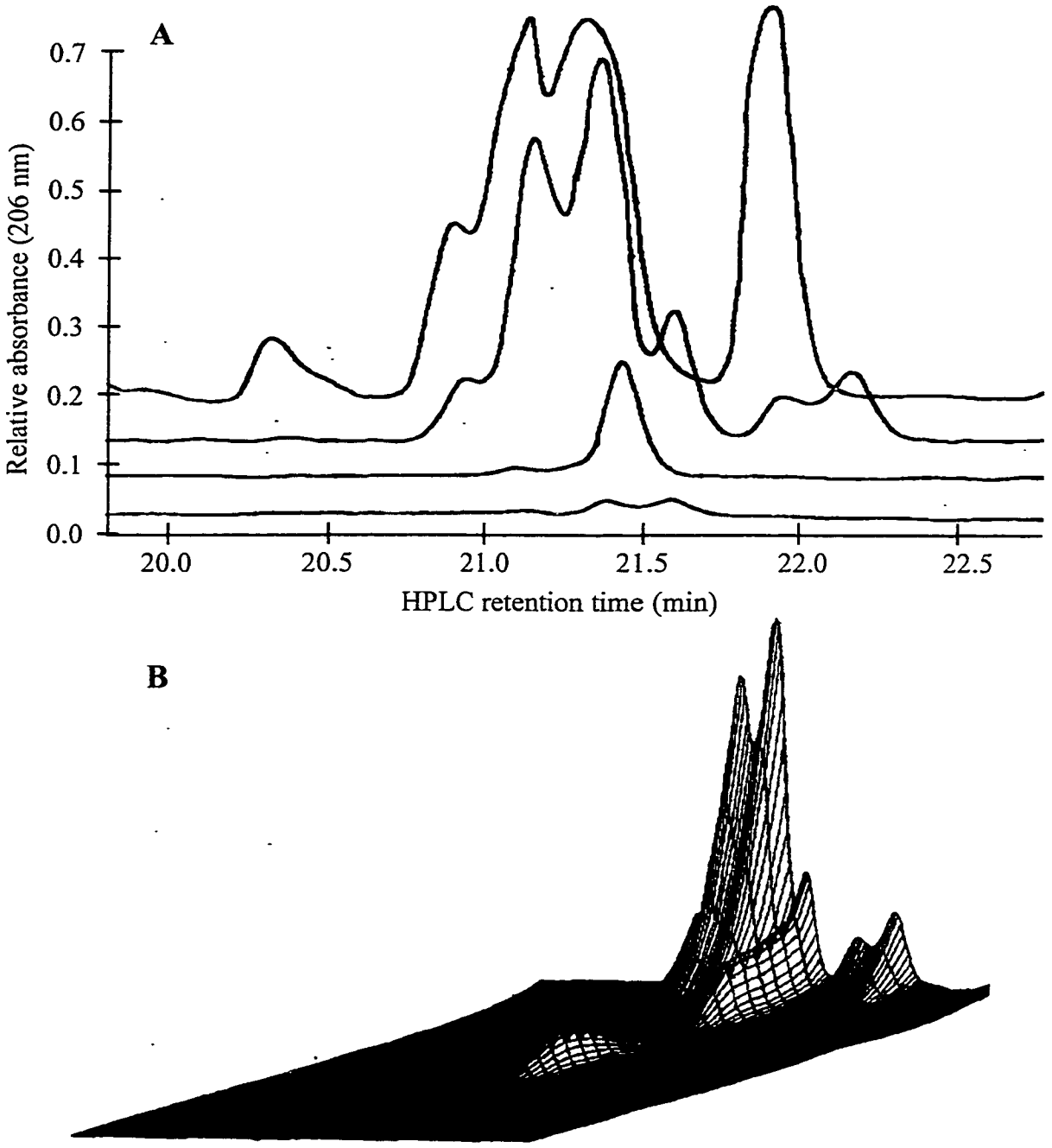


Figure 24. *In vitro* accumulation of LCOs in naringenin-induced *R. loti*/p2112 and *R. lupini*/p2112 (A) Relative absorbance at 206 nm of an overlay of HPLC-fractionated LCOs (from top to bottom): LCO mixture, naringenin-induced (5 μM) *R. loti*/p2112, naringenin-induced (5 μM) *R. lupini*/p2112, and non-induced. (B) Three dimensional plot of HPLC-fractionated LCOs from naringenin-induced *R. loti*/p2112.

observed in *R. lupini*/p2112. These multi-apex peaks do not appear in the analysis of non-induced cultures. Therefore, the LCOs accumulated in naringenin-induced FITA-harboring strains further complement the data obtained from the radiolabeled ones (Figure 12) in that they have similar retention times and relative intensities (i.e. *R. loti* > *R. lupini*). In addition, the retention times observed for LCOs in these elution profiles are similar to those of the radiolabeled LCOs shown in Figure 12. A three-dimensional plot is shown as an example of the spectral characteristics of the accumulated LCOs (Figure 24B).

Similar HPLC analyses of aldonic acid-induced wild-type *R. lupini* were carried out to assess of the *in vitro* accumulation of LCOs (Figure 25). The putative LCOs (Rt from 20.5 to 24 min) were collected for root hair deformation assays. In addition, HPLC analyses of isoflavone induced wild-type *R. lupini* were also carried out, although no LCO peaks were detected (data not shown).

E.6. ROOT HAIR DEFORMATION OF *LUPINUS ALBUS* ROOTS

HPLC fractionated LCOs (Rt from 20.5 to 24 min) from naringenin-induced *R. loti*/p2112 (Figure 24) or aldonic acid-induced *R. lupini* (Figure 25) were tested, after serial dilutions, for root hair deformation of *L. albus* roots incubated for 18-h. The results (Table 14) show that root hair deformation occured with the positive control (*R. loti*/p2112), as well as with both tetronic acid- and erythronic acid-induced *R. lupini* LCOs at dilutions of 10^{-4} and 10^{-6}. Such phenomenon were also observed, to a much lower extent, with the negative control, but only with a non-diluted LCO extract. However, no root hair deformation was observed with the succinic acid-induced LCOs. Deformation of root hairs, observed on *L. albus* roots was not extensive, i.e. less than 10 incidents per segment, and consisted of curled, Y-shaped or swollen root hairs. These events are similar to the ones reported by other investigators (e.g. Ardourel *et al.* 1994). However, they

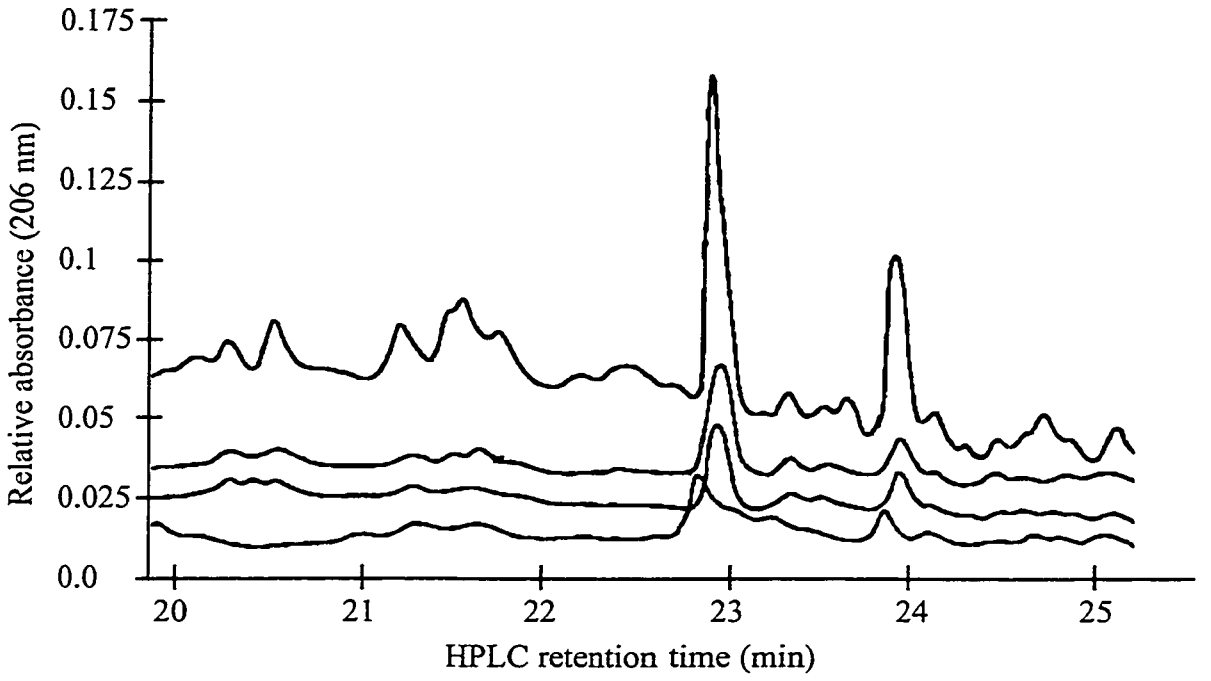


Figure 25. *In vitro* accumulation of LCOs in *R. lupini* after treatment with aldonic acids

(A) Relative absorbance at 206 nm of an overlay of HPLC-fractionated LCOs (from top to bottom): tetronic acid-induced (10 mM), erythronic acid-induced (20 mM), succinic acid-induced (20 mM), and non-induced *R. lupini*.

Table 14. Root hair deformation by LCOs on *Lupinus albus* roots[1,2]

Dilution	*Rhizobium loti*/p2112	Wild type *Rhizobium lupini*			
	Naringenin	none	EA	TA	SA
10^{0}	-	±	-	-	-
10^{-2}	-	-	-	-	-
10^{-4}	±	-	±	±	-
10^{-6}	+	-	+	+	-

[1]*L. albus* roots were treated with HPLC-purified LCOs of different dilutions, and root hair deformation was monitored after 18-h incubation. Symbols: -, ± and +, denote no root deformation observed, <5 or <10 incidents, respectively.

[2]Thirty-mL bacterial cultures were induced with either 5 μM naringenin, 20 mM erythronic acid (EA), 10 mM tetronic acid (TA), or 20 mM succinic acid (SA) for LCO accumulation.

could not be recorded as computerized images due to the lack of sufficient magnification (data not shown).

E.7. NATURAL OCCURENCE OF ERYTHRONIC AND TETRONIC ACIDS IN *LUPINUS ALBUS*

Whole seed effusates and root exudates of *L. albus* (cv Kievskij), as well as the cotyledon and seed coat tissues soaked in water for 24, 48, 72 and 96 h, were analyzed by HPLC for their aldonic acid content (Table 15). It should be noted that under the HPLC conditions used, the most polar compound of lupin isoflavones (2'-hydroxygenistein 7-*O*-glucoside) exhibits a retention time of 16.88 min, whereas erythronic and tetronic acids elute at 2.85 and 6.61 min, respectively. In addition, the spectral characteristics of the recovered aldonic acids are similar to the standard solutions used for generating their extinction coefficients (section E.1.1).

HPLC analysis of the different fractions (Table 15) shows that both seed effusates and root exudates contain significant amounts of tetronic and erythronic acids, with a predominance of the former compound over the latter. In fact, the amount of tetronic acid is 3 to 4-fold higher in the seed effusates, and an impressive 130-fold higher in root exudates, than those of erythronic acid (Table 15). It is interesting to note that the total amounts of aldonic acids, determined after Sep-Pack elution and EtOAc re-extraction of the medium, were found to exceed those of simple EtOAc extractions by 1.2 to 1.6-fold (Table 15). Assuming that Sep-Pack elution allowed their release from a molecular component, which otherwise was not possible with simple EtOAc extraction, this suggests that aldonic acids exist both in free and bound forms. The amounts of erythronic and tetronic acids found in the root exudates of 3-7 d old seedlings were found to be 8.9 ± 11% and 1175.7 ± 5% nmol·seed^{-1}, respectively. Whereas the amounts of both aldonic acids remain almost constant in the cotyledons for 96 h, a gradual decrease of both compounds is observed in the seed coats over the same period. This suggests that the cotyledons act as a reservoir of

Table 15. Erythronic acid and tetronic acid content in seed effusates, root exudates and seed parts of *Lupinus albus*[1]

A.	Seed effusates				Root exudates			
Extraction method[2]	EA		TA		EA		TA	
	nmol/ seed	SE[3] (%)	nmol/ seed	SE (%)	nmol/ seed	SE (%)	nmol/ seed	SE (%)
Sep-Pack (EtOAc ext.)	31.3	*	128.7	*	ND	-	ND	-
Sep-Pack (50% MeOH)	5.2	41	11.8	28	ND	-	ND	-
Total Sep-Pack ext's[4]	36.5	-	140.5	-	ND	-	ND	-
EtOAc extraction	30.8	6	90.2	6	8.9	11	1175.7	*
B. Seed parts	**Cotyledons**				**Seed coats**			
Age of seedlings Soaking time (h)	EA		TA		EA		TA	
	nmol/ mg f.wt	SE[3] (%)	nmol/ mg f.wt	SE (%)	nmol/ mg f.wt	SE (%)	nmol/ mg f.wt	SE (%)
24	98.3	*	137.9	11	14.3	15	34.4	29
48	108.7	*	83.0	14	9.3	21	24.7	23
72	69.8	17	126.1	27	5.1	35	19.8	30
96	100.4	*	145.9	*	4.2	32	18.5	36

[1]Duplicate HPLC analyses of *L. albus cv* Kievskij: whole seed effusates or root exudates (A) and of seed parts (B) were carried out.

[2]Seed effusates were either (a) pre-purified on C18 Sep-Pack whose wash was re-extracted with EtOAc, then the Sep-Pack eluted with 50% aq. MeOH, or (b) extracted with EtOAc.

[3]Standard errors (% of SE) of duplicate HPLC determinations; *, SE<5%.

[4]Total of the two C18 Sep-Pack fractions analyzed.

Abbreviations: EA, erythronic acid; TA, tetronic acid, ND, not determined; ext, extraction; f.wt, fresh weight.

these molecules, as compared with the seed coats which released ca. 50-70% of their content during the 4-d soaking (Table 15B). Therefore, it appears that the quantities of aldonic acids recovered in the seed effusates, root exudates and cotyledons are, on a molar basis, much higher than those of isoflavonoids, and amounted to 44-, 240- and 4000-fold higher values than total isoflavones, respectively (Gagnon *et al.* 1995b).

Similar analyses carried out with seed effusates of another lupin cultivar (Primovsky) indicated the presence of both aldonic acids, in a 2 to 3-fold higher amounts than the cv Kievskij (data not shown). These results suggest that the natural occurrence of aldonic acids in other *Lupinus spp.* may be a more common phenomenon in legume *spp.*

F. DISCUSSION

Symbiotic interactions between legumes and *Rhizobium spp.* contribute extensively to the overall nitrogen cycle via nitrogen fixation that takes place in the symbiotic organs, the root nodules. In addition, the importance of legume crops in agriculture has prompted researchers to investigate the molecular basis of species-specificity in these symbiotic interactions. The discovery of molecular signalling, by plant flavonoids and bacterial LCOs, has unravelled "the key to the legume door" (Spaink, 1992). This phenomenon has gained much interest in the field of molecular plant-microbe interactions to such an extent that it has been extended to other symbiotic systems. A good example is the symbiotic endomycorrhizae, which were reported to respond to flavonoid signals in a specific manner by an increased hyphal growth (Gianinazzi-Pearson *et al.* 1989). For these findings to be significant, however, the increased hyphal growth requires flavonoids and CO_2 (Poulin *et al.* 1993) and yet, the involvement of flavonoids has been questioned (Bécard *et al.* 1995).

The few flavonoid compounds that have previously been reported to act as species-specific signals in the Leguminosae, seem to establish a rationale for the various symbiotic systems (e.g. *Rhizobium spp.* vs legume hosts) that have been investigated. However, as additional flavonoid signals (Table 1) are being reported in justification of the strict species-specificity in symbiosis, such a phenomenon has become questionable. The specificity of the biological effects of LCOs has been combined with that of flavonoids in order to lend support to the rationale of species-specificity (Long, 1996). However, other molecules, such as betaines have recently been shown to act as specific signals (Phillips *et al.* 1992). In fact, the *nod* genes of *R. meliloti* are inducible with betaines, whereas they are not used by other bacterial species such as *R. loti* (Lopez-Lara *et al.* 1995). Therefore, flavonoids, betaines, as well as sulfated LCOs, are responsible for the species-specificity in the symbiosis between alfalfa and *R. meliloti.*

Of the several *Rhizobium*-legume interactions that have so far been reported, signalling in *Lupinus albus* and *R. lupini* symbiosis remains to be determined. This symbiotic

association may be characterized by the following features. *R. lupini* is an 'exceptional' symbiont, due to the physical location of its *nod* genes (Scott *et al.* 1996), and its copious secretion of exopolysaccharides (Figure 2B) as compared to other *Rhizobium spp.* In addition, the host (*L. albus*) exhibits a marginal symbiotic behaviour; its *Rhizobium*-infected nodules are neither determinate nor indeterminate, as is the case in most other legume *spp.*, and they are referred to as the 'crown nodules' of lupin (Hirsh, 1992). In addition, *L. albus* is one of the rare legumes that does not need to be endomycorrhized for optimal phosphorous uptake (Koide, 1991). But, above all, the bacterial symbiont has to cope with the capability of lupin roots which constitutively synthesize a variety of prenylated isoflavones (Gagnon *et al.* 1995b, and refs therein). These metabolites are known to act as antimicrobial compounds, rather than as *nod* gene inducers. However, during the course of this study, several lines of evidence, derived from both direct and indirect approaches, have tended to support a role for prenylated isoflavones as signalling molecules.

Studies of the differential, *in vitro* adsorption of lupin isoflavones showed that genistein monoprenyls, such as derrone, wighteone and lupiwighteone, were adsorbed at the highest rate onto *R. lupini* membranes. We have successfully applied this experimental approach to other *Rhizobium spp.* which revealed higher adsorption rates of flavonoid aglycones known to act as *nod* gene inducers, as compared with other weak-inducers or non-inducers. Therefore, the results obtained with lupin isoflavone adsorption onto *R. lupini* membranes suggest a role for these monoprenylated derivatives of genistein in this symbiotic system. The fact that isoflavone adsorption did not only increase with the increasing prenylation, demonstrates that the adsorption observed was not the result of increasing lipophilicity of the test compounds. The increased rate of secretion of *nod* gene inducers has been reported to occur in *Vicia sativa* roots after elicitation with the compatible symbiont (VanBrussels *et al.* 1990). A similar phenomenon has recently been demonstrated in lupin roots for the secretion of genistein monoprenyls after elicitation with

R. lupini (Gagnon and Ibrahim, 1997). This finding further substantiates a putative role for these isoflavones as *nod* gene inducers, based on their higher adsorption, as compared with the other compounds tested.

We have observed, in this study, significant effects of the medium composition on bacterial growth, as well as its response to isoflavonoid treatments. In contrast with the MA medium, the results obtained with the GS medium demonstrate more significant effects on bacterial growth, both stimulatory and inhibitory. Furthermore, these results corroborate those obtained with 10 μM isoflavones which inhibited the growth rate of *R. lupini* when cultivated in the complex LB medium (Gagnon, 1993).

Whereas the antimicrobial activity of phytoalexins of the Leguminosae, which are mostly prenylated pterocarpans, has been extensively documented (Tahara and Ibrahim, 1995 and refs therein), there are no published reports on the stimulatory effects of prenylated isoflavones on the growth of any *Rhizobium* strain. The most pertinent reported case is the effect of pre-incubation of *B. japonicum* with daidzein, its *nod* gene inducer, in abolishing the inhibitory effect of glyceollin I, a prenylated pterocarpan derived from daidzein (Parniske *et al.* 1991). In this study, the growth stimulation of *R. lupini* by prenylated isoflavones, such as the monoprenylated derivatives of genistein, licoisoflavone A and lupalbigenin constitute the first reported evidence for growth stimulation of a compatible symbiont by the constitutive compounds of its host. These results suggest that these compounds are putative *nod* gene inducers.

These two experimental approaches, although indirect, have provided evidence for the high rate of adsorption of lupiwighteone and derrone, as well as their stimulatory effect on the growth rate of the symbiont at such a low concentraion (0.5 μM), which renders them the most likely candidates as inducers of nodulation genes.

The use of the cosmid pRmM57 for the inducibility of *nodC::lacZ* fusions in *R. lupini*, as a direct approach, has not revealed a significant role for any of the lupin isoflavonoids tested as *nod* genes inducers. However, the fact that **(a)** 4-C heterocyclic molecules such

as the betaine, stachydrine (Phillips *et al.* 1992) have been shown to act as *nod* gene inducers in *R. meliloti*, and **(b)** because of the preliminary evidence for the natural occurrence of similar molecules in lupin tissues, but with an oxygen atom instead of a nitrogen (Figure 1), prompted us to test the aldonic acids, erythronic and tetronic, as inducers of *nod* genes in *R. lupini*. These experiments revealed a dose-dependent response of erythronic acid- and tetronic acid-induced *R. lupini*/pRmM57 and ß-galactosidase activity, but not after induction with succinic acid. Furthermore, lupiwighteone was found to act in synergy with erythronic acid albeit with low, but significant ß-galactosidase activity. These results strongly suggest that erythronic acid is a *nod* gene inducer of *R. lupini*. Furthermore, the synergistic effect of the latter compound with lupiwighteone constitutes the first evidence of *nod* gene induction in *Rhizobium* that is promoted by two, yet structurally different molecules. However, further investigation of the inducibility by tetronic acid will be required in order to account for any of heterologous biais. The natural occurence of aldonic acids in seed parts, seed effusates and root exudates provides further evidence for their role as co-inducers of *nod* genes in this symbiosis. Furthermore, the amounts of aldonic acids observed in these tissues are in agreement with those used for ß-galactosidase assays and LCO biosynthesis with *R. lupini*.

The requirement for betaine in the activation of the transcriptional regulator *nodD2* of *R. meliloti* has been well established (Phillips *et al.* 1992). As well, the activation of *nodD1* and *nodD3* by flavonoid inducers, such as luteolin, has been thoroughly investigated (Mulligan and Long, 1989), although that of *nodD2* is independent of luteolin (Honma *et al.* 1990). However, the induction of *nod* genes by a combination of betaines and flavonoids has not yet been investigated in *R. meliloti*. Considering the fact that *nodDs*, as transcriptional regulators, are homologous to the *LysR* family which act as heterodimers in their binding with the DNA promoter sequences, the latter species appear as a prime example to study the combined effects of betaines and flavonoids on the induction of *R. meliloti*.

A clear link has been established between *nod* gene induction and *Nod* factor biosynthesis (Carlson *et al.* 1994). Therefore, the inducibility of *nod* genes in *Rhizobium* by *nodC::lac Z* fusions is adequate to use as an experimental approach, since most inducers have been discovered using it as a complement to other approaches. Of the symbiotic systems where the flavonoid inducer has been determined, metabolic radiolabeling of LCOs as well as their accumulation, were achieved using the natural inducer. Both experimental approaches are complementary since they are used to investigate the same parameter, i.e. the induction of genes that encode the ezymes required for biosynthesis of LCOs (Carlson *et al.* 1994). Alternatively, FITA constructs have been used to by-pass the need to identify the natural inducer, while they allow the determination of the structure of the LCO produced. A good example is the case of *R. loti* where the structure of its *Nod* factor has been elucidated, but whose *nod* gene inducer remains to be determined (Lopez-Lara *et al.* 1995).

In contrast with the ease of discovering an inducer molecule for *R. loti* (tetronic acid), the incorporation of labeled precursors into LCOs of *R. lupini* has proven to be cumbersome. In fact, the various labeling experiments carried out with FITA-harboring *R. lupini* did not enable us to determine an unequivocally good LCO precursor. It has previously been reported that both ^{14}C-acetate and ^{14}C-glucosamine act as good precursors for the biosynthesis of LCOs in *Rhizobium spp.* (Spaink *et al.* 1992; Lopez-Lara *et al.* 1995). It has also been shown that metabolic radiolabeling of LCOs takes place preferentially with ^{14}C-glucosamine than ^{14}C-acetate (Price and Carlson, 1995). However, having used every radiolabeled precursor molecule available commercially, our results suggest that the LCOs of *R. lupini* may not be based on either glucosamine, galactosamine or mannosamine oligomers, as is the case for all *Nod* factors isolated to date (Dénarié *et al.* 1996). It is possible that an epimerase, acting on the amino sugars in *R. lupini*, may be responsible for the low levels of incorporation into *R. lupini* LCOs, especially when ^{14}C-glucosamine is used as a precursor. Our data suggest that this may be

the case, since low levels of radiolabeled LCOs were obtained in *R. loti,* when ^{14}C-galactosamine was used as a precursor, eventhough the *R. loti* LCOs are known to consist of glucosamine oligomers (Lopez-Lara *et al.* 1995).

Nonetheless, a relatively high level of labeled LCOs has been achieved using higher amounts of labeled glucosamine, coupled with a more efficient extraction method, which resulted in a dose dependency between the level of induction and LCO biosynthesis in wild type *R. lupini.* In addition, the identity of the inducible LCOs has been confirmed by hydrolysis with specific enzymes, as well as by their ability to impart root hair deformation on the compatible host. These methods are considered satisfactory for the preliminary characterization of LCOs (Price and Carlson, 1995; Ardourel *et al.* 1994). We have used two additional enzymes: *N*-acetylglucosaminidase which acts on both glucosamine and galactosamine oligomers, and proteinase K which serves as a negative control for hydrolysis, since LCOs do not contain peptide bonds.

Furthermore, we have demonstrated, for the first time, the induction of *nod* genes in both *R. meliloti* and *R. loti*, with tetronic acid, as well as its ability to promote the metabolic radiolabeling of LCOs. In addition, their identity was established by hydrolysis with specific enzymes. Taken together, these results provide strong evidence for its role as a signal molecule. These phenomena were not observed in other *Rhizobium spp.* investigated such as *B. japonicum*, *R. leg.* bv *viciae* and *R. leg.* bv *trifolii*.

In conclusion, our results demonstrate, for the first time, that tetronic acid acts as an inducer of *nod* genes in two related *Rhizobium spp.* (*R. lupini* and *R. loti*), as well as in *R. meliloti*, although the natural occurrence of tetronic acid has yet to be investigated in the compatible hosts of *R. meliloti* and *R. loti* (*Medicago* and *Lotus spp.*, respectively). Taken together, the results reported in this work may render the phenomenon of species-specificity by flavonoids in *Rhizobium*-legume interactions a questionable 'doctrine', at this point.

G. PERSPECTIVES FOR FUTURE WORK

Several questions have arisen during the progress of this work that merit further investigation. The following avenues of research are yet to be explored in order of priority.

1. The structural determination of both of the aldonic acids that were found in lupin-seed effusates and root exudates. Gas chromatography-mass spectral (GC-MS) analysis, as well as proton- and ^{13}C-NMR spetroscopy could be used.

2. The aldonic acid-induced LCOs of *R. lupini* should be isolated on a preparative scale, and their components generated by chemical/enzyme degradation, analyzed by GC-MS analysis. The latter studies will resolve the identity of the amino sugar of the oligomeric backbone, and allow the use of the specific precursor molecule for the production of radiolabeled LCOs in *R. lupini*.

3. Investigation of the *nod* genes of *R. lupini* using a molecular approach, especially those encoding the putative epimerase activity, as well as that of chitin synthase (i.e. *nodC*).

4. Since tetronic acid has been shown to be a putative inducer of *nod* genes in *R. meliloti*, investigation of its effects, in combination with luteolin, will provide further evidence for the synergistic effect of two structurally distinct molecules that may exist in this system.

H. CLAIM TO ORIGINALITY AND CONTRIBUTION TO KNOWLEDGE

The experimental work reported in this thesis has made original contributions in the field of host-symbiont interactions. The following are some of the novel contributions, hietherto reported for the first time.

1. The discovery that erythronic and tetronic acids act as natural *nod* gene inducers and their occurence in lupin tissues, and hence as signal molecules in the *L. albus-R. lupini* symbiosis.

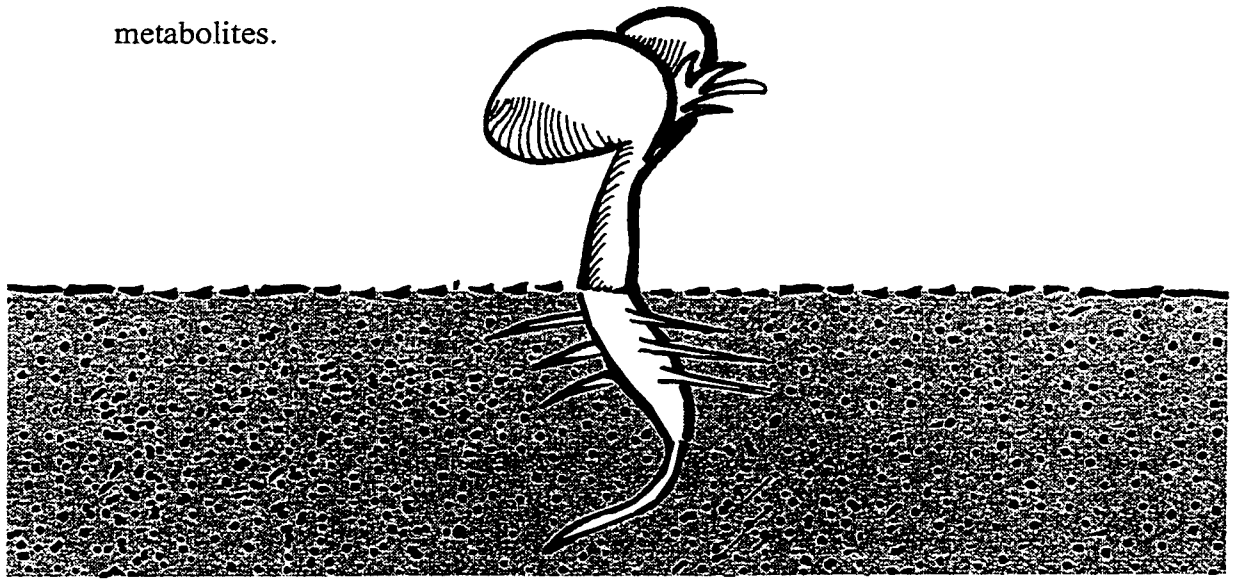
2. The evidence of a synergy between two structurally distinct molecules (monoprenylated isoflavones and aldonic acids) that enhances *nod* gene expression in *R. lupini.*

3. The reported action of prenylated isoflavones as *nod* gene inducers.

4. The evidence for a significant *in vitro* growth stimulation of a *Rhizobium sp.* by prenylated isoflavonoids.

5. The discovery that a synthetic aldonic acid, tetronic acid, promotes *nod* gene induction in three *Rhizobium spp.* (*R. lupini*, *R. loti* and *R. meliloti*).

6. The profiling of indirect, but novel, methods for the screening of *nod* gene inducers in leguminous species that contain a wide variety of pre-infectional secondary metabolites.



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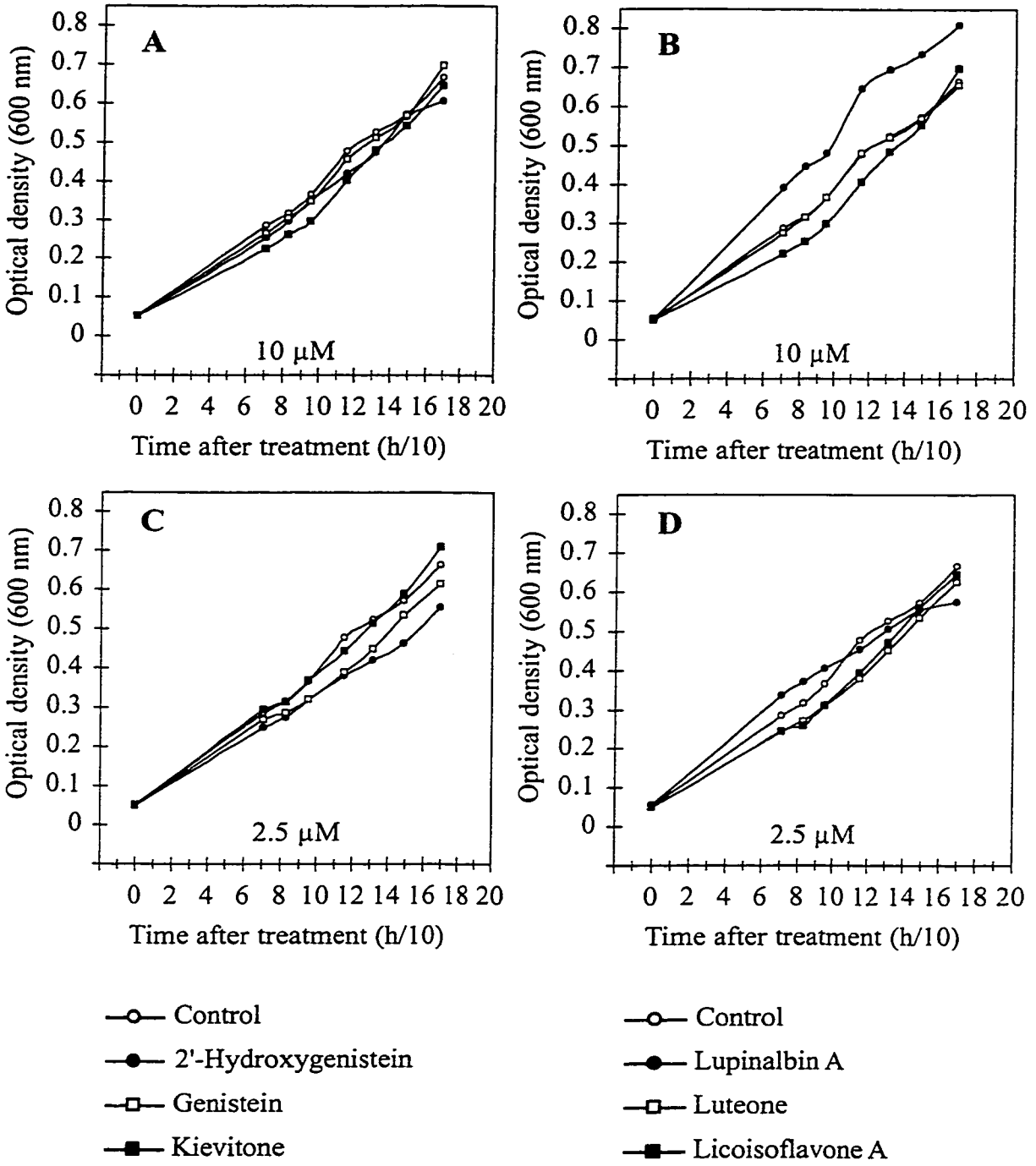
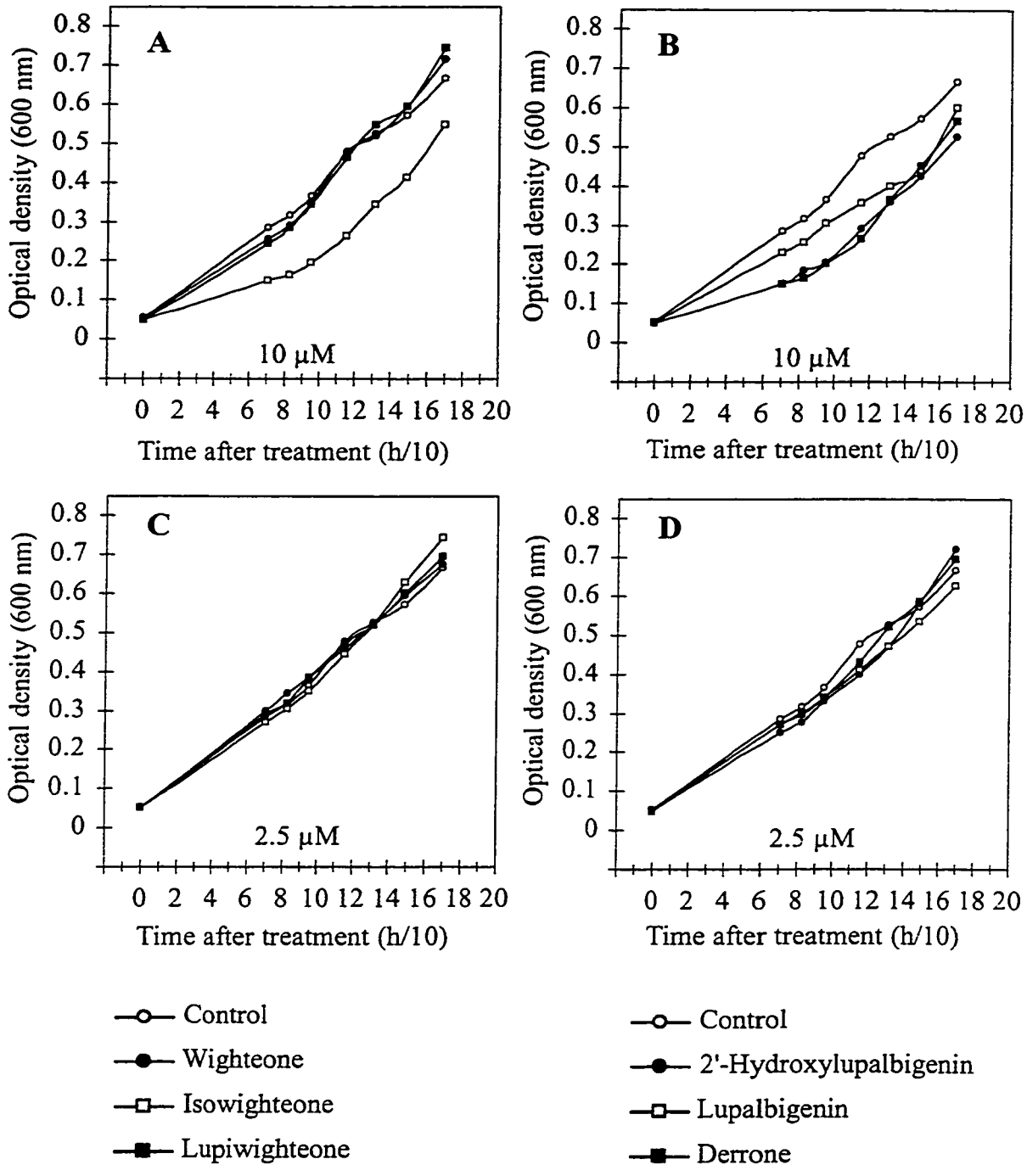
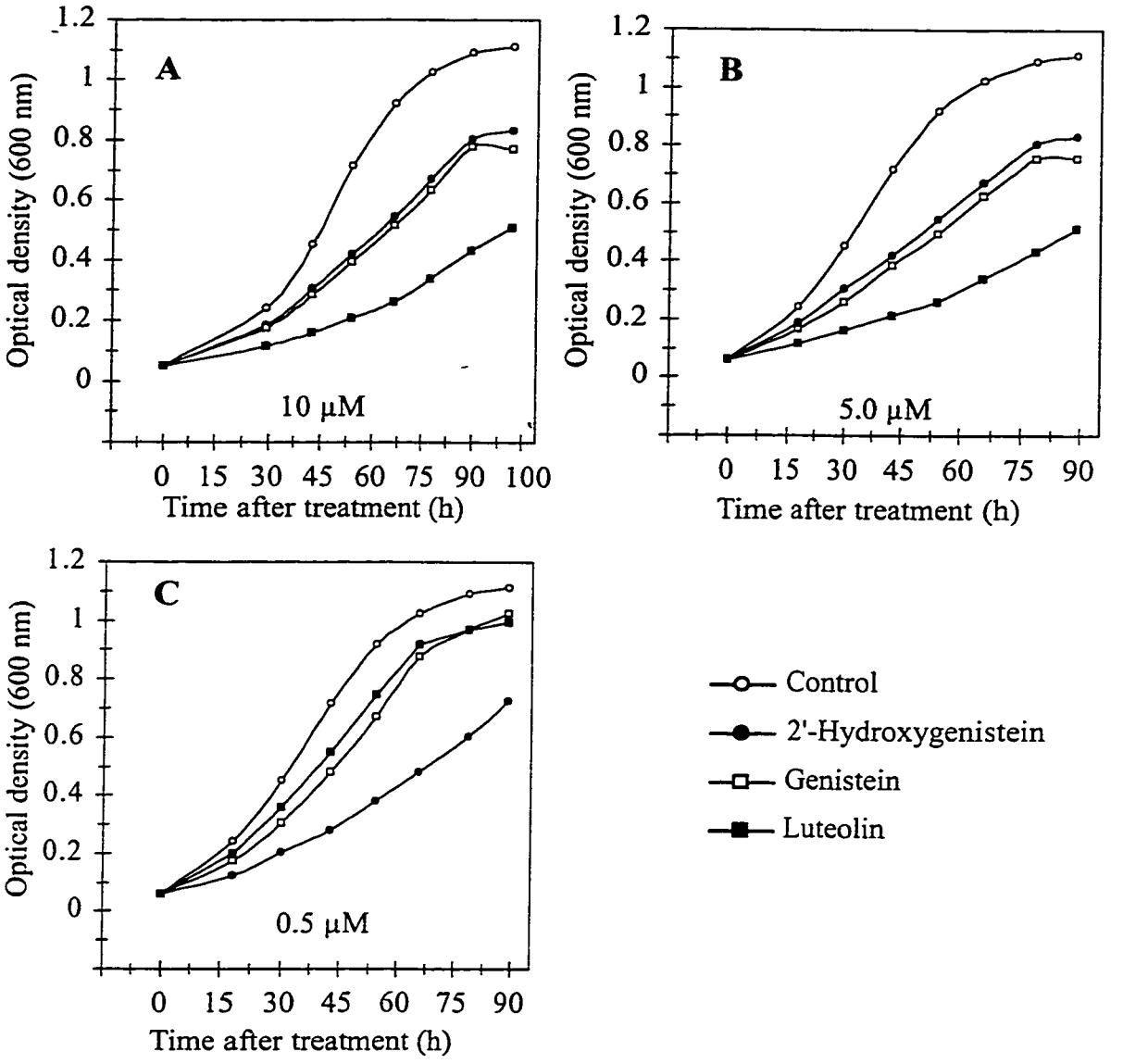
J. APPENDICES



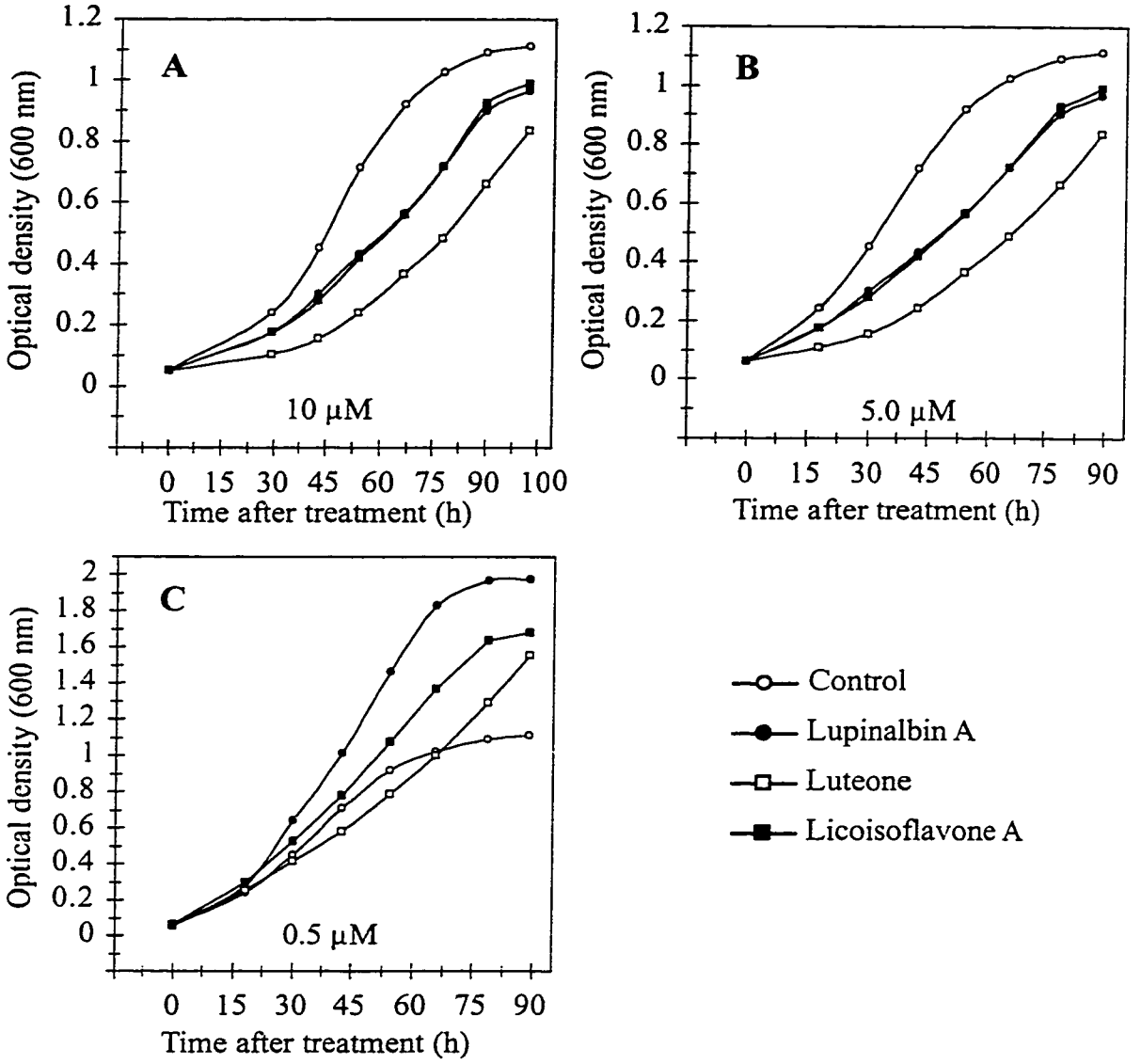
Appendix I. Growth of *Rhizobium lupini* in MA medium at 28°C; after treatment with 10 µM (A,B) and 2.5 µM (C,D); genistein, 2'-hydroxygenistein and kievitone (A,C); and lupinalbin A, luteone and licoisoflavone A (B,D), the optical density (OD_{600}) of a 1/5 dilution of the bacterial cultures was measured at different time intervals.



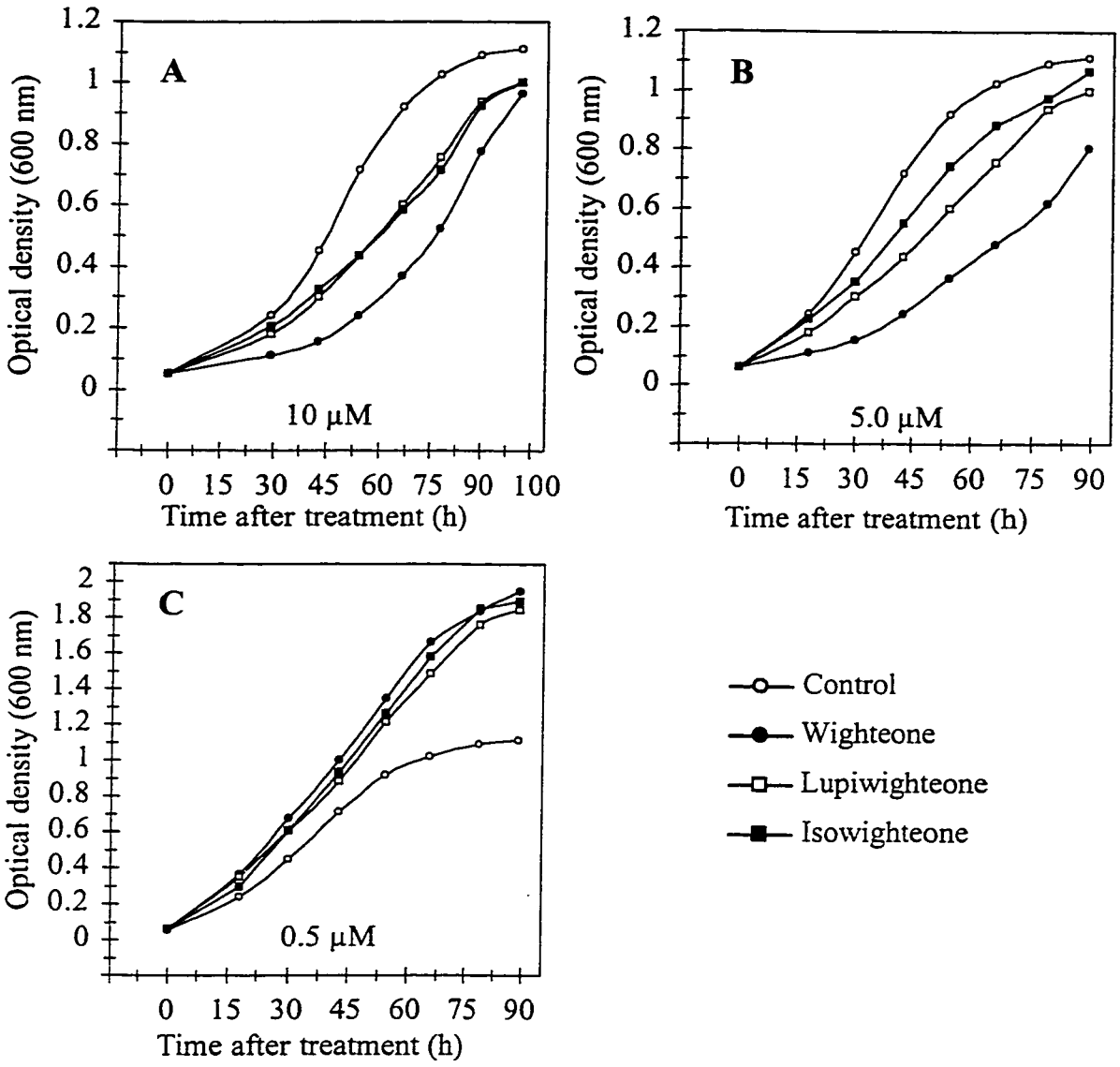
Appendix I (cont'd). Growth of *Rhizobium lupini* in MA medium at 28°C; after treatment with 10 µM (A,B) and 2.5 µM (C,D); of wighteone, lupiwighteone and isowighteone (A,C); and of 2'-hydroxylupalbigenin, lupalbigenin and derrone (B,D), the optical density (OD_{600}) of a 1/5 dilution of the bacterial cultures was measured at different time intervals.



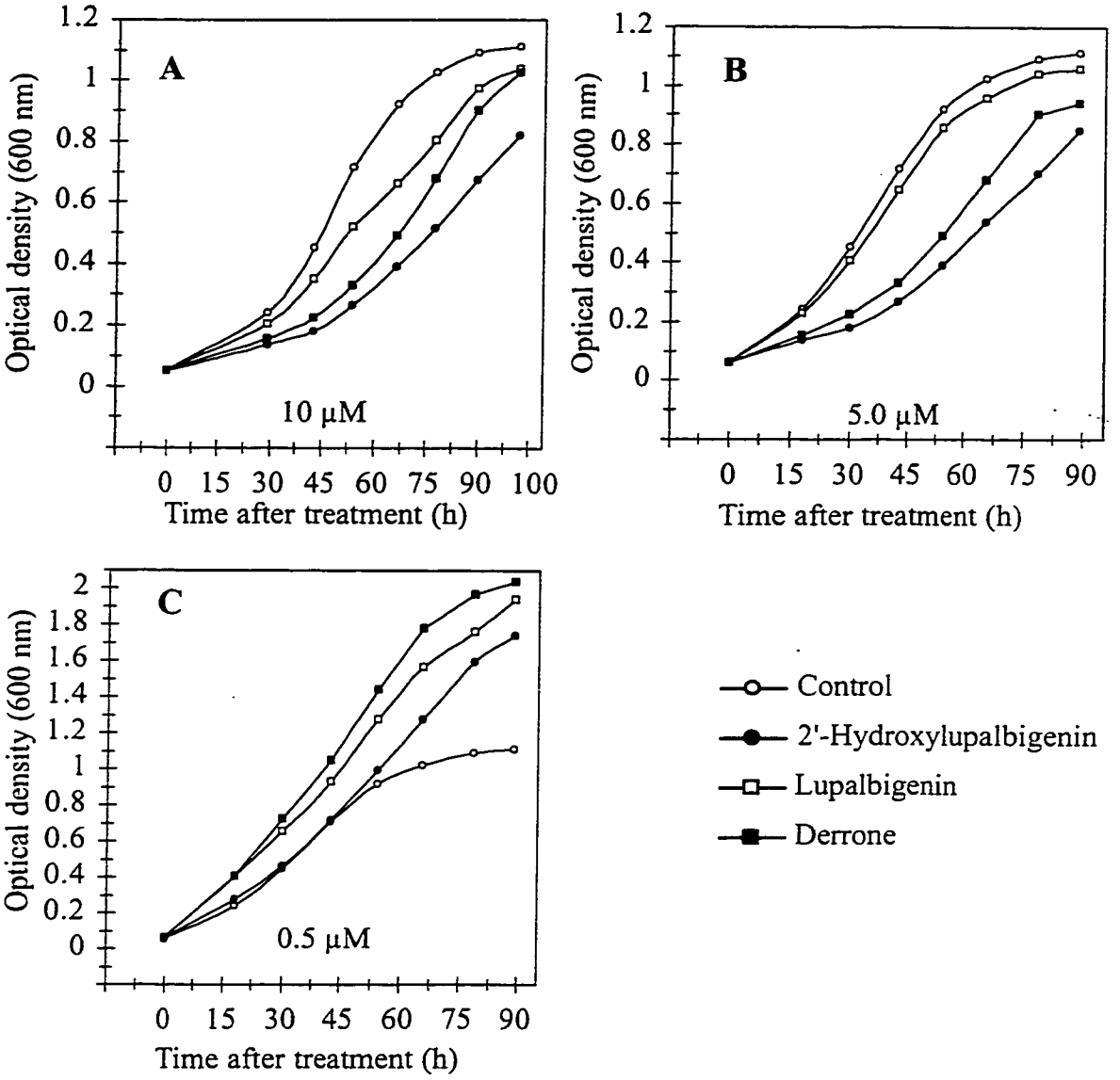
Appendix II. Growth of *Rhizobium lupini* in GS medium at 28°C; after treatment with 10 µM (A), 5.0 µM (B) and 0.5 µM (C), of genistein, 2'-hydroxygenistein and luteolin, the optical density (OD_{600}) of a 1/5 dilution of the bacterial cultures was measured at different time intervals.



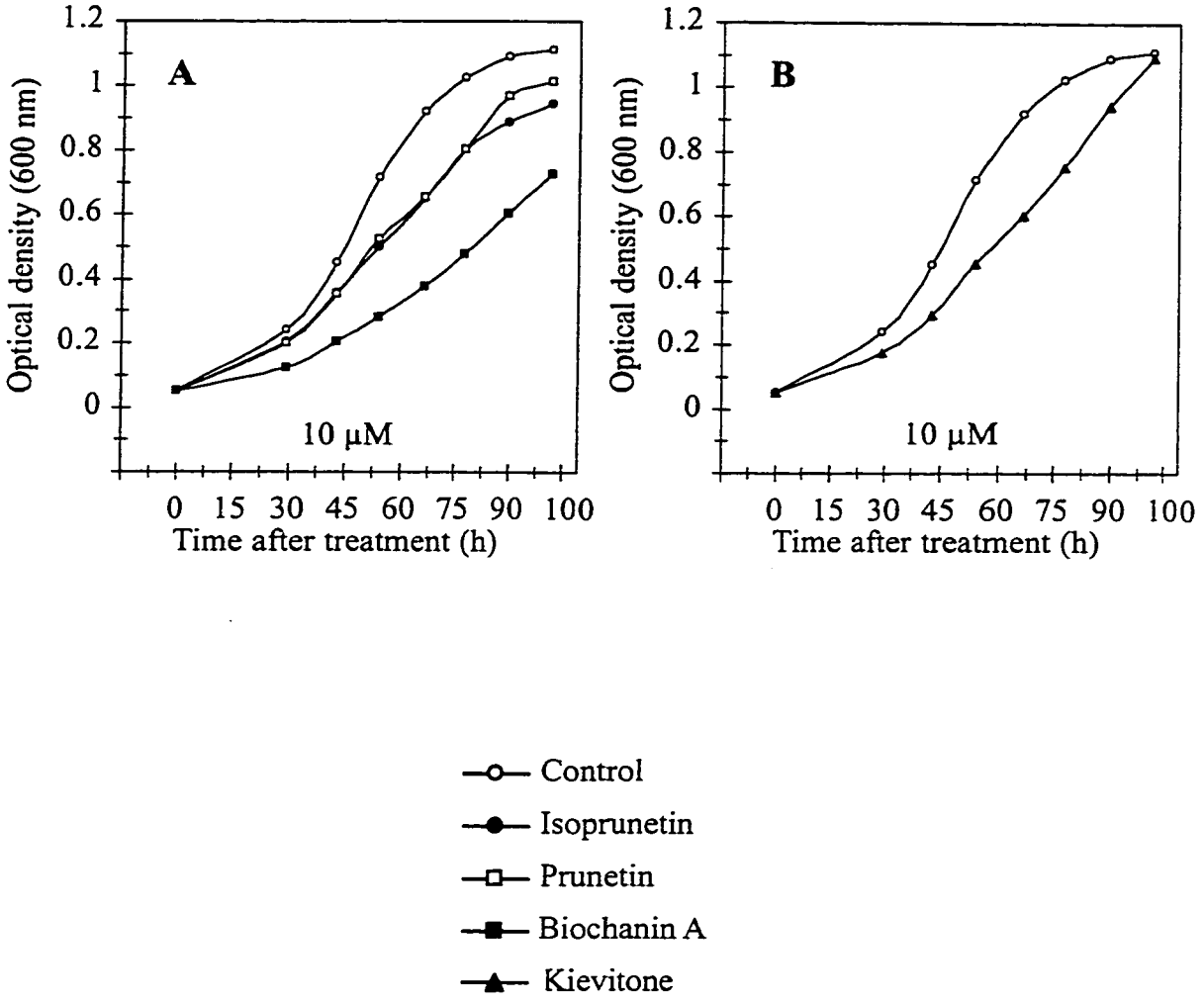
Appendix II (cont'd). Growth of *Rhizobium lupini* in GS medium at 28°C; after treatment with 10 µM (A), 5.0 µM (B) and 0.5 µM (C), of lupinalbin A, luteone and licoisoflavone A, the optical density (OD_{600}) of a 1/5 dilution of the bacterial cultures was measured at different time intervals.



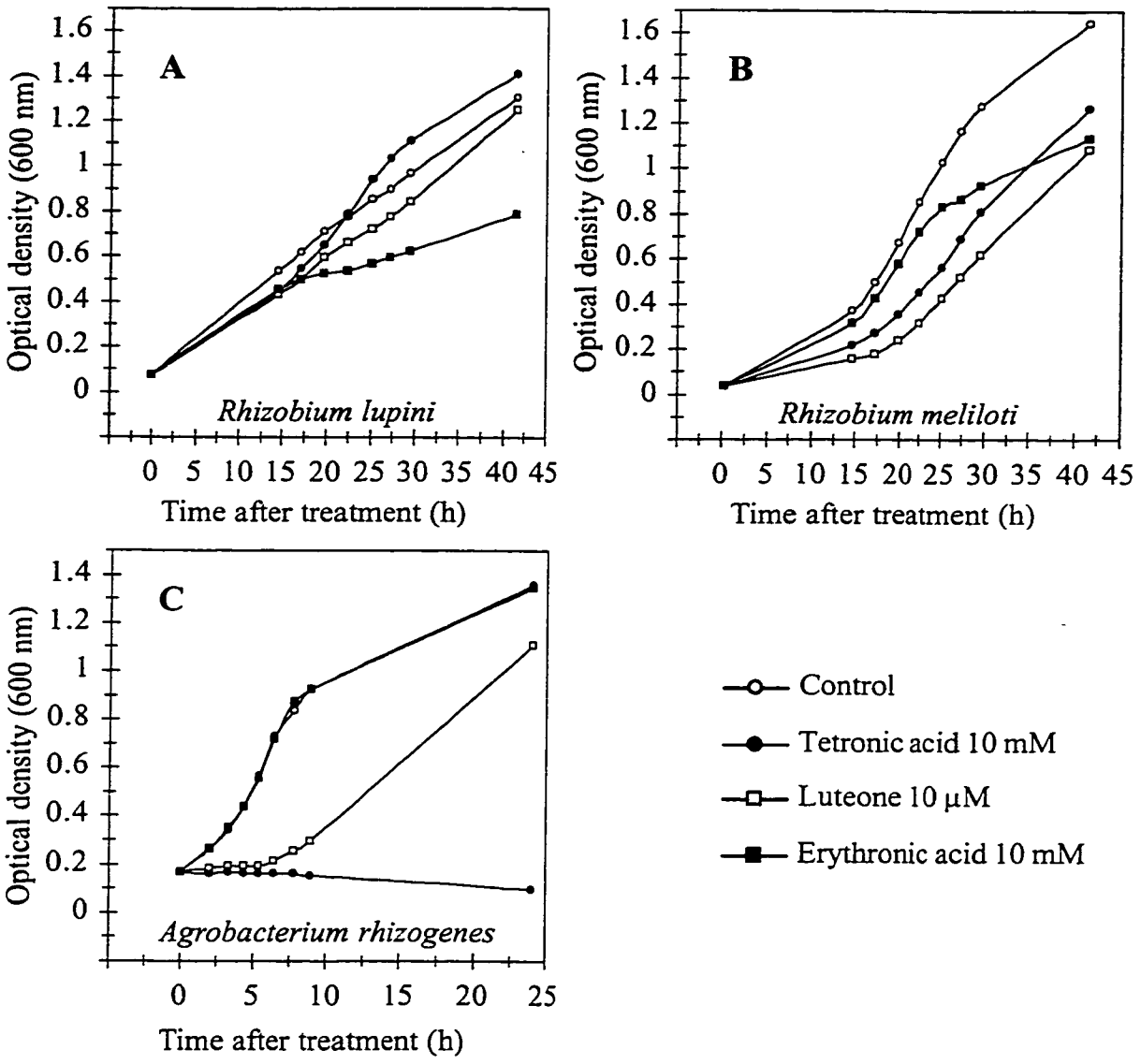
Appendix II (cont'd). Growth of *Rhizobium lupini* in GS medium at 28°C; after treatment with 10 µM (A), 5.0 µM (B) and 0.5 µM (C), of wighteone, lupiwighteone and isowighteone, the optical density (OD_{600}) of a 1/5 dilution of the bacterial cultures was measured at different time intervals.



Appendix II (cont'd). Growth of *Rhizobium lupini* in GS medium at 28°C; after treatment with 10 µM (A), 5.0 µM (B) and 0.5 µM (C), of 2'-hydroxylupalbigenin, lupalbigenin and derrone, the optical density (OD_{600}) of a 1/5 dilution of the bacterial cultures was measured at different time intervals.



Appendix II (cont'd). Growth of *Rhizobium lupini* in GS medium at 28°C; after treatment with 10 µM isoprunetin, prunetin, biochanin A (A), and kievitone (B), the optical density (OD_{600}) of a 1/5 dilution of the bacterial cultures was measured at different time intervals.



Appendix III. Growth of *Rhizobium lupini* (A), *Rhizobium meliloti* (B) and *Agrobacterium rhizogenes* (C) in YM medium; at 28°C (*Rhizobium*) and 22°C (*Agrobacterium*); after treatment with 10mM tetronic acid, 10 µM luteone and 10 mM erythronic acid, the optical density (OD_{600}) of a 1/5 dilution of the bacterial cultures was measured at different time intervals.