The Isoflavonoids of *Lupinus albus*, Their Constitutive and Environmentally Induced Accumulation and Secretion, and Their Effect on Bacterial Growth.

Hubert Gagnon

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ABSTRACT

The Isoflavonoids of *Lupinus albus*, Their Constitutive and Environmentally Induced Accumulation and Secretion, and Their Effect on Bacterial Growth.

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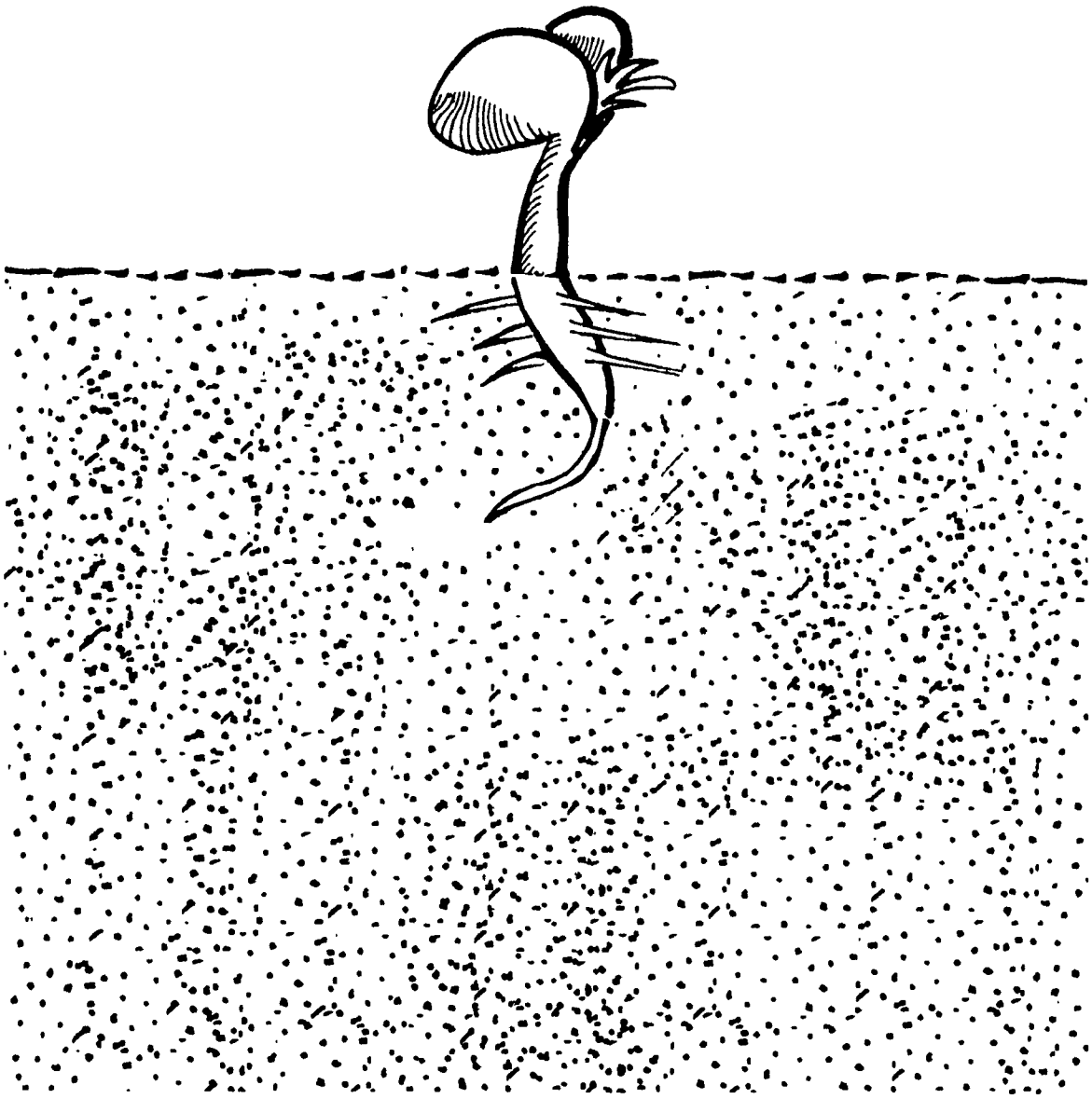
The accumulation of lupin isoflavonoids and their release in the exudate were studied in sterile seedling parts during early stages of germination. The isoflavonoid content of lupin seedlings stressed with their symbiont (*Rhizobium lupini*), as well as with biotic and abiotic elicitors has also been investigated. To further corroborate the results, radiolabeled *trans*-cinnamic acid was used as precursor to demonstrate their biosynthesis *in vivo*. In addition, the effect of various flavonoid compounds on the growth of different bacterial species was determined. The results show active synthesis of these metabolites in all parts analyzed from day 2 to 4, resulting mainly in the accumulation of glucosylated isoflavonoids. This was followed by a gradual decrease in the isoflavonoid content of all parts analyzed mostly at the expense of glucosides. Secretion of isoflavonoids by sterile root systems was mainly in the form of genistein monoprenyls at all sampled stages. These derivatives accumulated and were secreted predominantly when the seedlings were stressed with their symbiont (*R. lupini*), whereas other biotic and abiotic elicitors resulted mainly in the accumulation and secretion of 2'-hydroxygenistein monoprenyls. Root nodules, which are the symbiotic organs, were shown to accumulate mostly diprenylated derivatives. The latter compounds were also found in significantly high amounts in seedling parts and their occurrence suggests that they may play a role in development. The results are discussed in relation to the roles these isoflavonoids may play in developmental, stress and symbiotic processes in *L. albus*.

À Colette, Paul et Lise



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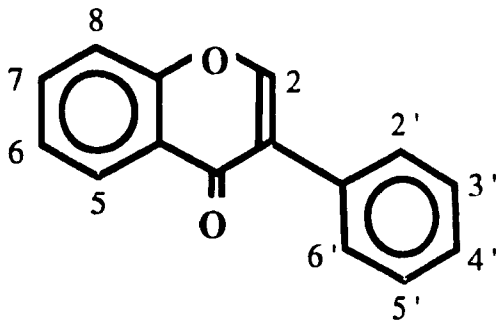
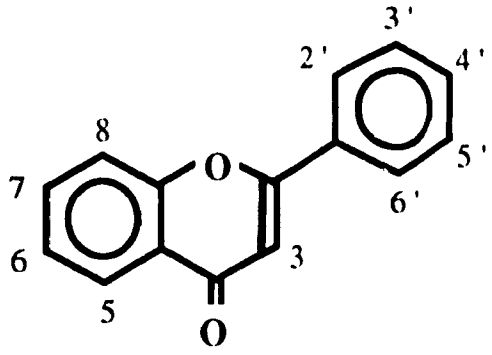


Isoflavone numbering system



Flavone numbering system

Compound Name	Generic Name
Derrone	7,8-Hydroxypyrano-5,4'-dihydroxyisoflavone
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
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
Quercetin	3,5,7,3',4'-Pentahydroxyflavone
Wighteone	6-prenyl-5,7,4'-Trihydroxyisoflavone

LIST OF ABBREVIATIONS

Abbreviation	Name
bv	biovar
B.	*Bradyrhizobium*
DMSO	Dimethyl sulfoxide
HOAc	Acetic acid
HPLC	High pressure liquid chromatography
l.	*Leguminosarum*
MeOH	Methanol
µV	Microvolt
NaOAc	Sodium acetate
OD	Optical density
RH	Relative humidity
R.	*Rhizobium*
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume

A. INTRODUCTION

Flavonoid compounds constitute one of the most ubiquitous secondary metabolites originating from the phenylpropanoid pathway. These plant phenolics include several classes of related ring structures with different oxidation levels, such as the flavanones, flavones, flavonols and isoflavones (Heller and Forkmann, 1988). Flavonoids have been assigned a number of roles as protection against UV radiation (Li *et al.*, 1993), antioxidants (Middleton and Kandswami, 1992 and refs. therein), phytoalexins (Dixon *et al.*, 1983; Smith and Banks, 1986; Ebel and Grisebach, 1988), regulators of polar auxin transport (Jacobs and Rubery, 1988), inducers of pollen germination and development (Mo *et al.*, 1992; Taylor and Jorsensen, 1992) and more recently as molecular determinants of species specificity for symbiotic nitrogen fixation in the Leguminosae (Long, 1989). This broad spectrum of roles illustrates their involvment in the survival and environmental fitness of plants.

This dissertation will border on three main aspects of *Lupinus albus* isoflavonoids, namely their possible implication in plant development, their role as phytoalexins and finally, their involvement in the establishment and control of symbiosis with *Rhizobium*.

It has been well established that plants respond to various stress factors in the environment, including pathogenic microorganisms, although successful infections are rare. Confronted with infection, plants have evolved an array of defense mechanisms, such as lignification and the hypersensitive response (Collinge and Slusarenko, 1987), among others (Lamb *et al.*, 1989). Lignification will take place after wounding or pathogenic stress in order to form a physical barrier. The hypersensitive response will lead to the killing of the infected cells, via phytoalexin accumulation to phytotoxic levels, in order to prevent propagation of the infection (Collinge and Slusarenko, 1987). A well

characterized response is the *de novo* synthesis and accumulation of secondary metabolites known as phytoalexins (Paxton, 1981; Dixon *et al.*, 1983; Smith and Banks, 1986). These molecules confer resistance to various pathogens. Isoflavonoid phytoalexins have been reported in several species of the Leguminosae (Ingham, 1983; Smith and Banks, 1986). It is known that most, if not all, legumes have the ability to synthesize such secondary metabolites in response to fungal attack, or elicitation with biotic and abiotic elicitors (Albersheim and Valent, 1978; Darvill and Albersheim, 1984; Smith and Banks, 1986; Ebel and Grisebach, 1988).

Another characteristic feature of leguminous species is their association with the symbiotic bacteria of the genus *Rhizobium* (Smith and Douglas, 1987). This symbiotic interaction enables the plant to fix atmospheric nitrogen via the formation of specialized plant organs, the root nodules (Long, 1989). These symbiotic interactions contribute extensively not only to organic nitrogen content of plants, but also to the productivity of soils and are, therefore, of significant ecological importance (Krishnan and Pueppke, 1992).

The study of these symbiotic interactions has become an intensive field of research in the past decade. One of the findings of this research has been the recognition that some flavonoid compounds act as molecular signals synthesized by the host plant to activate bacterial infection (Table 1; Long, 1989; Nap and Bisseling, 1990). These signals were found to act as chemoattractants of *Rhizobium* (Caétano-Annolès *et al.*, 1988a), as well as inducers of bacterial *nod* genes (Peters *et al.*, 1986). In addition, other flavonoid compounds were found to act as chemorepellants for *Rhizobium* (Caétano-Annolès *et al.*, 1988a), and inhibit the expression of *nod* genes (Peters and Long, 1988). Furthermore, there exists a differential synthesis, along the root axis of both inducer and inhibitor molecules (Redmond *et al.*, 1986).

It was only recently, that studies have started to focus on host gene expression and secondary metabolite pools in response to *Rhizobium* infection. An outcome of this

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

Host latin name	Host common name	Symbiont	Flavonoid inducer	Flavonoid inhibitor	Ref. or source
Lupinus albus	White lupin	*R. lupini*	ND	ND	--
Lupinus albus	White lupin	*B. lupini*	ND	ND	--
Lupinus luteus	Yellow lupin	*B. lupini*	ND	ND	--
Medicago sativa	Alfalfa	*R. meliloti*	Luteolin	Apigenin	1, 2, 3
				Naringenin	2, 3
				Eriodictyol	2, 3
Trifolium repens	White clover	*R. l.* bv *trifolii*	Luteolin	Daidzein	4
			7,4'-DiOH-flavone;	Formononetin	4
					4
			4'-OH-7-methoxyflavone		4
Trifolium repens	White clover	*R. meliloti*	7,4'-DiOH-flavone	Daidzein	4
				Genistein	4
				Kaempferol	4
Glycine max	Soybean	*R. fredii*	Daidzein	ND	5
Glycine max	Soybean	*B. japonicum*	Daidzein	ND	6
Phaseolus vulgaris	Common bean	*R.l.* bv *phaseoli*	Delphinidin	ND	7
			Petunidin		7
			Malvidin		7
Pisum sativum	Pea	*R. l.* bv *viceae*	Apigenin	ND	6
			Eriodictyol		6
Vicia villosa	Vetch	*R. l.* bv *viceae*	ND	ND	--

1, Mulligan and Long, 1985; 2, Peters *et al.*, 1986; 3, Firmin *et al.*, 1986; 4, Redmond *et al.*, 1986; 5, Sadowsky *et al.*, 1988; 6, Kosslak *et al.*, 1987; 7, Hungria *et al.*, 1991a. ND, not determined.

research shift, from symbiont to host, has been to extend the role of flavonoids beyond host recognition in these symbiotic interactions (Okker *et al.*, 1992; Hirsch, 1992). One of the facts which tends to support this view is that the spectrum of secondary metabolites synthesized by the host is altered upon bacterial attachment (Recourt *et al.*, 1992; Schmidt *et al.*, 1992). In addition, nodule formation is accompanied by an increase in phytoalexin synthesis, which seems to correlate with the stages of nodule maturation, i.e. the ability of nodules to fix nitrogen (Karr *et al.*, 1992). These facts allowed further understanding of the role of flavonoid compounds in plants, while enhancing our knowledge of the phenomenon of symbiosis in legumes.

It is interesting to note that members of the Leguminosae will respond to microorganisms, whether pathogen or symbiont, by producing chemical signals of flavonoid nature. Yet, pathogen infection may, or may not, elicit production of flavonoids that are different from those implicated in *Rhizobium* infection. Legumes may have evolved specific strategies to control flavonoid synthesis as means of coping with environmental stresses, or with *Rhizobium* symbiosis.

Lupinus albus (white lupin, Figure 1), a member of the Leguminosae, is an important crop species in East European and Asian countries. The phytochemistry of its isoflavonoids has been well studied (Figure 2; Harborne *et al.*, 1976; Igham *et al.*, 1983; Tahara *et al.*, 1984a; 1985a; 1989b; 1990; Shibuya *et al.*, 1991). *Lupinus albus* synthesizes a variety of isoflavonoid glucosides, aglycones, mono- and diprenylated derivatives, as well as a number of furano and pyrano derivatives of its prenylated isoflavones and coumaronochromones. All of the latter compounds originate from the same precursor, genistein, a simple 5-hydroxyisoflavone (Hagmann and Grisebach, 1984; Tahara *et al.*, 1984a). In contrast with the majority of legumes, white lupin constitutively synthesizes this variety of isoflavonoids, some of which exhibit effective antipathogenicity (Harborne *et al.*, 1976).

Figure 1: Photograph of *Lupinus albus*

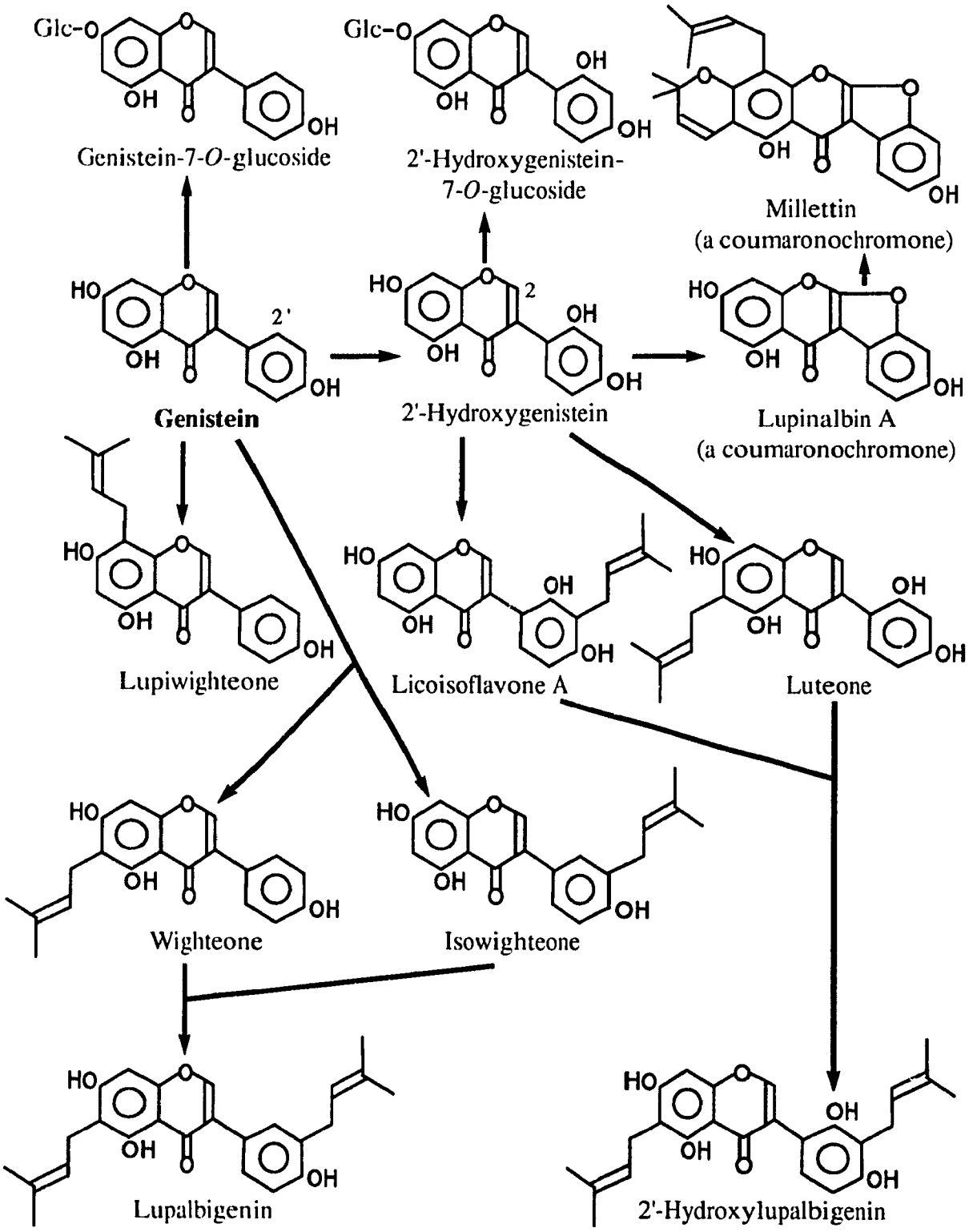


Figure 2: Molecular structure of lupin isoflavonoids

White lupin is capable of establishing a symbiosis with *Rhizobium lupini* * and *Bradyrhizobium lupini* (Table 1; Smith and Douglas, 1987). In contrast with several other *Rhizobium*-legume associations, the flavonoid signals which confer specificity to lupin symbiosis with *Rhizobium lupini* and *Bradyrhizobium lupini* are yet to be identified. In addition, the fact that the biosynthesis of prenylated isoflavones is constitutively expressed in lupin seems to confer a strong pathogen resistance to this species (Harborne *et al.*, 1976). These two features make lupin a very attractive candidate for the study of its isoflavonoid composition in relation to symbiosis with *Rhizobium* and pathogen resistance. Indeed, while similar recognition features should apply to the *Lupinus-Rhizobium* symbiotic interaction, this bacterium has yet to cope with the lupin endogenous pool of pre-infectional isoflavonoids. Therefore, white lupin is considered the system of choice to study the role of isoflavonoids as modulators of control over pathogenic and symbiotic organisms, and more generally in response to environmental stresses. Since the isoflavonoid pools in white lupin root system, as well as those compounds exuded into the rhizosphere may vary in response to various stresses, it is therefore relevant to analyze them, both qualitatively and quantitatively, during different stages of seed germination and seedling development.

* Listed as *R. loti* in the ATCC catalog, but since it was isolated from *Lupinus* species, it is designated as *Rhizobium lupini* throughout the text.

B. AIMS OF WORK

The aim of this project were:

(a) To develop a chromatographic protocol for the separation of glucosides, aglycones and prenylated derivatives of lupin isoflavonoids, in a single run, which is not available at the present time.

(b) To demonstrate the constitutive expression of isoflavonoids in lupin via incorporation of a radiolabeled precursor. Other incorporation studies have been conducted with seedlings that were stressed with elicitors.

(c) To study the distribution of isoflavonoids in the different organs of lupin as well as the root exudates in relation to different developmental events.

(d) To study the effects of stress factors (*R. lupini*, biotic and abiotic elicitors) on isoflavonoid accumulation and secretion. Alterations in isoflavonoid composition caused by biotic and abiotic elicitors will be compared to those caused by *Rhizobium* infection.

(e) To determine the isoflavonoid composition of root nodules in comparison with that of lateral roots from which they originate.

(f) To determine if lupin isoflavonoids affect the growth rate of *R. lupini*. Growth rates of *R. lupini* in a complex medium will be determined from growth curves. The latter will be compared with those of other bacterial species, in order to correlate the inhibitory effects of flavonoids on bacterial growth.

This project will contribute to our knowledge of the biological roles of isoflavonoids in relation to stress factors and symbiosis of white lupin, since very little work has been conducted on white lupin with respect to these aspects. Several questions will, therefore, be addressed and experimentally verified as a preliminary approach to this original, experimentally unknown, legume-*Rhizobium* symbiotic system. Furthermore, studies of the variation in the distribution of isoflavonoids in unstressed control seedlings during growth may help reveal their possible role in lupin development.

C. REVIEW OF LITERATURE

In this section, I will discuss both phenomena, phytoalexins and symbiosis, with the view to emphasize the implications of flavonoids in these interactions which involve both plant response mechanisms. In order to do so, I will first introduce the historical and general concepts of phytoalexins with examples found in the Leguminosae (Section C.1.1.). In addition, the phenylpropanoid pathway (Section C.1.2.), the synthesis of isoflavonoids (Section C.1.3.), and their prenylation will be briefly described (Section C.1.4.), as well as the experimental tools utilized for their study (C.1.5.). Furthermore, the elicitors responsible for phytoalexin accumulation (Section C.1.6.) and the effect of the latter on microorganisms (Section C.1.7.) will be discussed. Finally, the isoflavonoids that were identified from white lupin will be presented (Section C.1.8.).

In the second part, *Rhizobium*-legume symbiotic interactions will be introduced. This will include the classification and distribution of *Rhizobium* species (Section C.2.1.), the infection process (Section C.2.2.), their symbiotic genes (Section C.2.3.), and more specifically, the nodulation (*nod*) genes. A discussion of the *nod* genes structure (Section C.2.4.), the regulation of their expression (Section C.2.5.), their role in the establishment of symbiosis (Section C.2.6.), as well as the developmental relationship between the nodules and secondary roots (Section C.2.7.) will be presented in some detail. Finally, the features relative to host control on nodulation (Section C.2.8.) will be discussed in order to illustrate the role of flavonoids as modulators of control in legume-*Rhizobium* interactions.

C.1. FLAVONOID BIOSYNTHESIS AND PHYTOALEXINS

C.1.1. Historical and General Concepts

The original definition of phytoalexins included all molecules resulting from a defense mechanism, but did not refer to a chemically defined class of compounds (Smith and

Banks, 1986). These compounds were termed phytoalexins in 1940 by Muller and Borger, as they reported the first case of phytoalexin accumulation, a sesquiterpenoid (later shown to be rishitin) which conferred resistance of potato cultivars suceptible to *Phytophthora infestans*, a phytopathogenic fungus (Muller and Borner, 1940; Sato *et al.*, 1971). Twenty years later, the pterocarpan pisatin was isolated from pea pods in response to fungal infection (Cruickshank and Perrin, 1961). Its structure was determined as the first reported isoflavonoid phytoalexin. During the two decades that followed, a number of other phytoalexins (Figure 3) were reported belonging to various classes of compounds, including other flavonoids and pterocarpans (glyceollin), terpenoids (rishitin, capsidiol), stilbenes (resveratrol), polyacetylenes (wyerone), dihydrophenanthrenes (orchinol; Figure 3; Albersheim and Valent, 1978; Dixon *et al.*, 1983). It should be noted, however, that phytoalexin accumulation is not the only defense mechanism which confers resistance to plants (Darvill and Albersheim, 1984; Lamb *et al.*, 1989).

As the isolation of new phytoalexins progressed, many compounds were shown to be flavonoids (e.g. betagarin, a flavanone that accumulates post-infetionally in sugar beets; Figure 3; Richardson, 1981), as well as isoflavonoids that were isolated in most cases. Well documented examples are phaseollin from elicited common bean (Cruickshank and Perrin, 1963), and the 4 isomers of glyceollin from elicited soybean (Figure 3; Burden and Bailey, 1975; Lyne *et al.*, 1976). Such discoveries prompted the search for more isoflavonoid phytoalexins (Smith and Banks, 1986).

A working redefinition of phytoalexins was made in 1981, by the NATO Advanced Study Institute, to include "low molecular weight, antimicrobial compounds that are synthesized by and accumulated in plants after exposure to microorganisms" (Paxton, 1981). This definition implies that phytoalexin accumulation is classically post-infectional, and arises from remote precursors, so that *de novo* synthesis became a prerequisite for isoflavonoids to be associated with phytoalexins (Moesta and Grisebach, 1981b; Dixon, *et al.*, 1983; Smith and Banks, 1986). In fact, most legumes do not constitutively express the

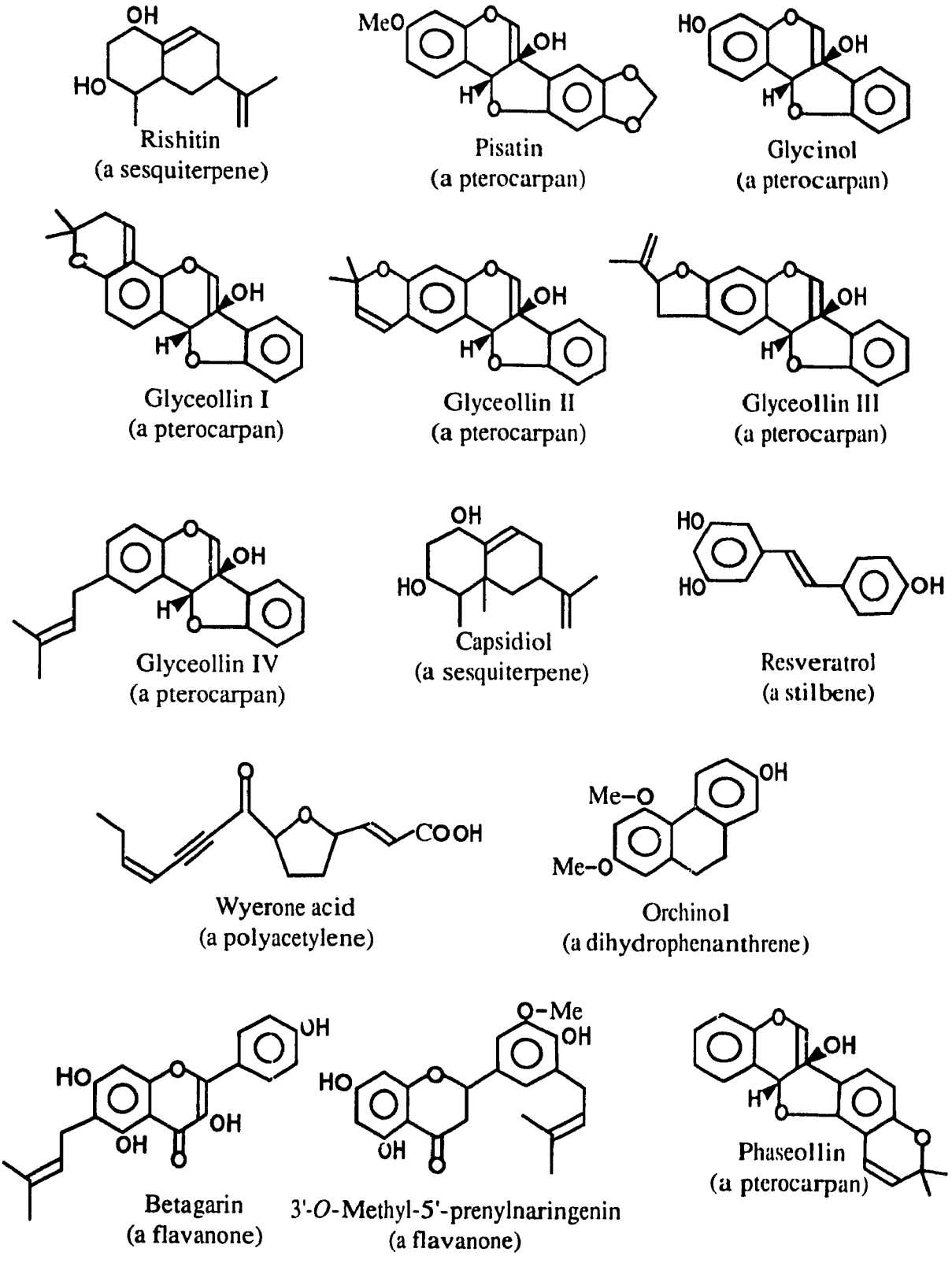


Figure 3: Some common phytoalexins

genes responsible for phytoalexin synthesis. However, there is still a debate about the extent of this definition, as Smith and Banks point out: "it is often a division of convenience in orienting research programs, but not always of functional significance" on assessing *de novo* synthesis as opposed to constitutive, pre-infectional synthesis of anti-fungal compounds (Harborne *et al.*, 1976; Smith and Banks, 1986). A good example of pre-infectional compounds is found in *Lupinus* species, which constitutively accumulate luteone (Fukui *et al.*, 1973; Harborne *et al.*, 1976) as well as several other prenylated coumaronochromones and isoflavones (Ingham *et al.*, 1983; Tahara *et al.*, 1984a), some of which are fungitoxic (Figure 2).

C.1.2. General Phenylpropanoid Pathway

Various plant phenolic constituents originate mainly from the deamination of L-phenylalanine by the action of L-phenylalanine ammonia lyase (PAL, E.C.4.3.1.5.), resulting in the formation of *t*-cinnamic acid (Figure 4; Koukol and Conn, 1961). It was demonstrated in several studies that both phenylalanine and *t*-cinnamic acid are equally good precursors of flavonoids (Hess and Schwochau, 1969; Hess *et al.*, 1971; Dixon and Fuller, 1977).

Trans-cinnamic acid is further hydroxylated into *p*-coumaric acid, a reaction catalyzed by cinnamate 4-hydroxylase (CA4H, E.C.1.14.13.11.). *p*-Coumaric acid represents a biosynthetic branching point between benzoic acids and other cinnamate-derived phenolics. *p*-Coumaric acid is then esterified by 4-hydroxycinnamate: CoA ligase (4CL, E.C.6.2.1.12.) with coenzyme A to yield 4-hydroxycinnamoyl-CoA. Following this step, a biosynthetic branching occurs between lignins and flavonoids (Figure 4; Ebel and Halhbrock, 1982; Heller and Forkmann, 1988).

Flavonoid compounds originate from the condensation of two precursors, 4-hydroxycinnamoyl-CoA and malonyl-CoA (incorporated as acetate units). The first committed step in flavonoid biosynthesis is catalysed by chalcone synthase (CHS,

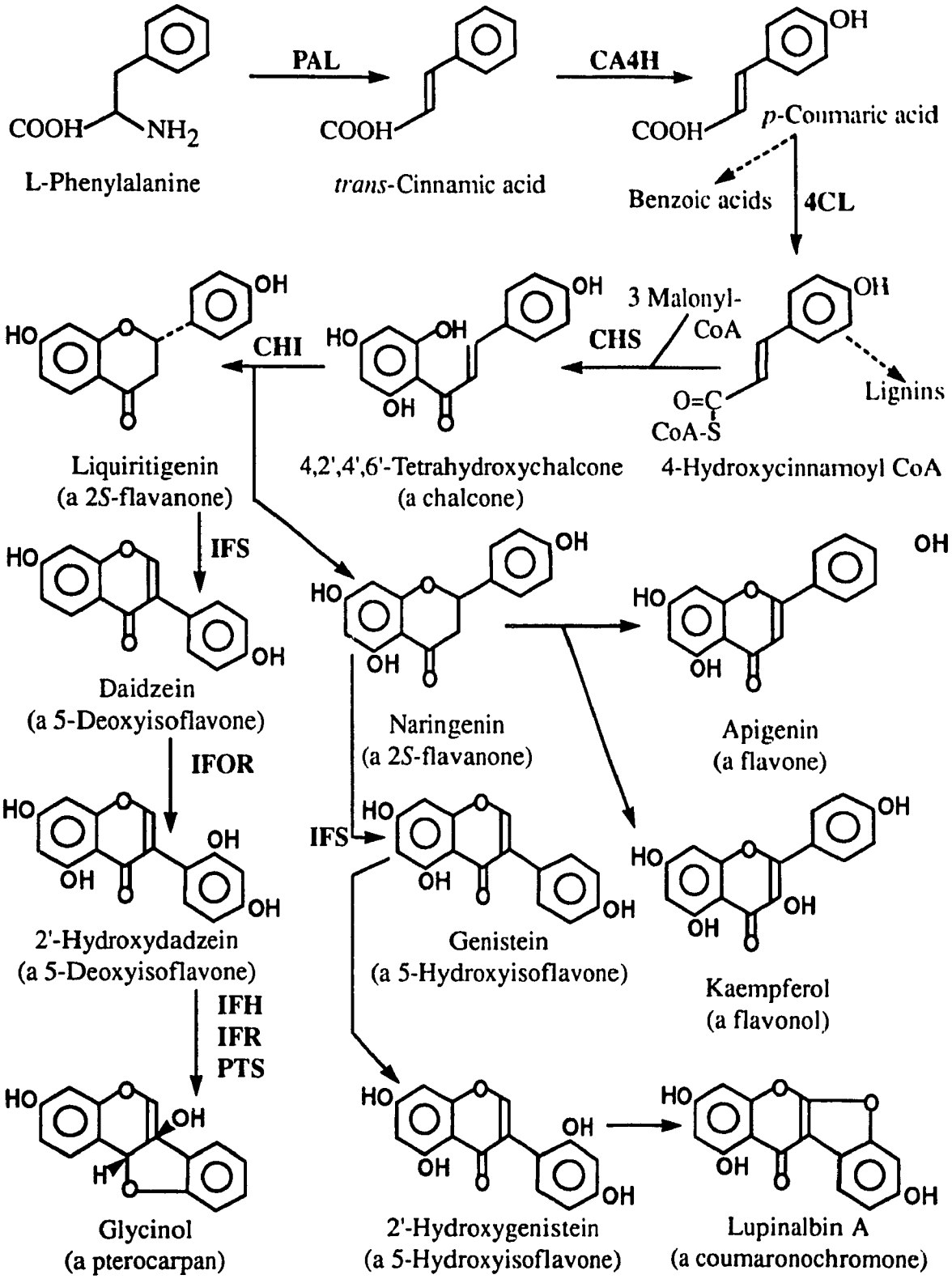


Figure 4: Flavonoid pathway

E.C.2.3.1.74.), where 3 molecules of malonyl-CoA contribute to ring A formation, whereas the phenyl group of 4-hydroxycinnamate contributes to the formation of ring B. The resulting C_{15} chalcone intermediate (4,2',4',6'-tetrahydroxychalcone) undergoes stereospecific rearrangement and C-ring closure by chalcone isomerase (CHI, E.C.5.5.1.6.), to yield the first flavonoid, naringenin, a (2S)-flavanone (Figure 4; Heller and Forkmann, 1988).

Two types of CHI enzymes are known which differ in their substrate specificity, for 5-deoxy or 5-hydroxy substituted chalcones, giving rise to liquiritigenin and naringenin, respectively (Hagmann and Grisebach, 1984). Following this step, a biosynthetic branching occurs between flavonoids and isoflavonoids, whose ring structures are based on a 1,3-diphenyl propane nucleus for flavonoids, or a 1,2-diphenyl propane nucleus for isoflavonoids (Ingham, 1983).

C.1.3. Isoflavonoid Biosynthesis

Isoflavonoids are derived by an aryl migration of ring B from C2 to C3 of the flavanone, and is catalyzed by a two step reaction (Kochs and Grisebach, 1986). It involves the action of isoflavone synthase (IFS), a cytochrome P-450 dependant monooxygenase, which yields a stable 2-hydroxyisoflavanone intermediate. The latter is further converted to genistein (5-hydroxy-) or dadzein (5-deoxyisoflavone) by a 2-dehydratase and a C2-C3 double bound formation (Hashim *et al.*, 1990). These two isoflavone aglycones (Figure 4) are the precursors of all other isoflavonoids (Hagmann and Grisebach, 1984; Kochs and Grisebach 1986).

Further rearrangement or substitution of the C- and B-rings, give rise to the major isoflavonoid subgroups including: isoflavanones, pterocarpans, and coumaronochromones, as well as the rotenoids, isoflavans, and coumestans (Heller and Forkmann, 1988). Isoflavanones are derived from their corresponding isoflavone precursors by the action of isoflavone oxidoreductase (IFOR; Tienmann *et al.*, 1987).

Pterocarpans are derived from 5-deoxy aglycones via three enzymatic reactions. First, the aglycone is transformed to its 2'-hydroxy derivative by an isoflavone 2'-hydroxylase (2'-IFH; Daniel *et al.*, 1990). Subsequent steps involve the formation of an isoflavanone intermediate by the action of isoflavone reductase (IFR), followed by pterocarpan synthase (PTS) that catalyzes the condensation of the 2'-OH with the reduced carbonyl group of the C ring (Bless and Barz, 1988). Pterocarpans are of common occurrence as phytoalexins, especially in the Leguminosae (Ingham, 1983). Similar to pterocarpans, it is expected that coumaronochromones are derived from the condensation of isoflavones 2'-hydroxyl group with carbon 2 (Figure 4).

Isoflavonoid aglycones may undergo further substitution via *O*-glycosylation, *O*-methylation, hydroxylation, acylation (mostly via malonylation of glucosides), or prenylation giving rise to the wide variety of isoflavonoids found in nature (Ingham, 1983). All of the enzymes discussed so far have been extensively reviewed by Heller and Forkmann (1988), Barz and Welle (1992), and by Ibrahim and Varin (1993).

The distribution of isoflavonoids is more or less restricted to the Papilionoidae sub-family of the Leguminosae, since 90% of these compounds are known to be produced by members of this group. Eventhough the isoflavonoids are restricted to this relatively large sub-family, they remain one of the most diverse classes of flavonoids, with well over 500 characterized aglycones and their derivatives (Ingham, 1983).

C.1.4. Prenylation of Isoflavonoids

Isoflavonoid phytoalexins include examples of various structural diversity starting with the simple prenylated isoflavone, luteone (Harborne *et al.*, 1976), the pterocarpan, glycinol (Weinstein *et al.*, 1981), the prenylated pterocarpan, glyceollin IV (Zärhinger *et al.*, 1979), the furano and pyrano derivatives of prenylated pterocarpans, glyceollins I, II and III (Figure 3; Cruickshank and Perrin, 1961), and the prenylated coumaronochromone, millettin (Figure 2; Tahara *et al.*, 1985a). Whereas the pterocarpan glycinol, isolated from

$CuCl_2$-treated soybean cotyledons, is fungitoxic to *Phytophthora megasperma* and is bacteriostatic to six bacterial species (Weinstein *et al.*, 1981), the introduction of a 6-dimethylallyl (prenyl) substituent on 2'-hydroxygenistein (luteone) can lead to a considerable increase in fungitoxicity (Harborne *et al.*, 1976; Welle and Grisebach, 1991). Other such prenylations confer fungitoxicity to flavanones as well. For example, 3'-*O*-methyl-5'-prenylnaringenin isolated from *Erythrina bertroana* (a legume tree) is fungitoxic to *Cladosporium cucumerinum* (Maillard *et al.*, 1987). In addition, methylation of pterocarpans (e.g. pisatin) can confer fungitoxicity to *Nectria haematococca* (Sweigard *et al.*, 1986).

Isoflavone synthase (Hagmann *et al.*, 1984; Kochs and Grisebach, 1986; Bonhof *et al.*, 1986) and isoflavonoid prenyltransferases (Biggs *et al.*, 1987; Laflamme *et al.*, 1993) are membrane associated and thus require microsomal preparations for their assay. In the latter reaction, the prenyl donor is dimethylallyl-pyrophosphate (DMAPP; Schröeder *et al.*, 1979; Zärhinger *et al.*, 1979). Prenyltransferases catalyse reactions leading to increased hydrophobicity of the substituted flavonoid (Gnanamackinam and Mansfield, 1981), as opposed to glycosylation which renders the substituted flavonoid more polar (Shibuya *et al.*, 1991). Due to the membrane localization of these enzymes, further investigations focused on the properties of the solubilized enzyme (Welle and Grisebach, 1991), and no prenyltransferase has yet been purified to homogeneity (Ibrahim and Varin, 1993).

C.1.5. Experimental Tools Utilized for the Study of Flavonoids

In the past decade, there has been a significant progress achieved in elucidating flavonoid biosynthesis. This is mainly due to the availbility of ^{14}C labelled precursors, the improvement of flavonoid enzyme preparations and enzyme assays, and more recently, the availability of cDNA probes for a number of genes coding for flavonoid enzymes (Dangl *et al.*, 1989). Consequently, our knowledge of the enzymology of the flavonoid pathway has been greatly extended (Ibrahim and Varin, 1993).

The rapid induction of PAL and CHS activities has been demonstrated in soybean hypocotyls and cell cultures after elicitation with cell wall preparations of *Phytophthora megasperma* (Borner and Grisebach, 1982). The availability of CHS and PAL cDNA clones made it possible to correlate the rapid and transient increase of CHS and PAL enzyme activities with increases in their mRNAs (Schmelzer *et al.*, 1984; Chapple and Hahlbrock, 1984; Ryder *et al.*, 1984). This well illustrates that the genes coding for these two key enzymes are expressed after elicitation, and that other enzymes are responsible for phytoalexin synthesis in this system. In addition, the regulation of phytoalexin synthesis appears to be at the transcriptional level. In fact, it was shown that inhibitors of mRNA synthesis did suppress accumulation of the phytoalexin glyceollin in soybean (Chapple and Hahlbrock, 1984; Ebel and Grisebach, 1988).

It has been recently demonstrated that CHS mRNA of soybean is encoded by a multigene family. The screening of a genomic library with CHS cDNA has revealed 6 different genes by RFLP analysis, and extensive homology was found among the aligned sequences (Wingender *et al.*, 1989). These results showed that soybean responds differently to stresses, including elicitation, so that they demonstrate the complexity of CHS gene regulation in different organs, and the varying levels of expression with respect to various stresses (Wingender *et al.*, 1989). Furthermore, experiments with *Phaseolus vulgaris* indicated that this regulatory system responds in a discriminate manner to pathogens (e.g. *Agrobacterium*) and symbionts (e.g. *Rhizobium*, see later; Ryder *et al.*, 1987).

High Performance Liquid Chromatography (HPLC) has also enabled the reliable identification and quantification of flavonoid compounds. The most commonly used HPLC protocols for the elution of flavonoids are carried out in isocratic modes (Pietta *et al.*, 1989; Ferrer *et al.*, 1990; Biggs *et al.*, 1987; Wolff and Werner, 1990; Tomas-Lorente *et al.*, 1989; Zaat *et al.*, 1989; Tomas-Lorente *et al.*, 1990). However, when the analyses are made on extracts containing a wide range of polarities (e.g. variously substituted

flavonoids), the use of elution gradients on HPLC becomes more appropriate (Mulligan and Long, 1985; Graham, 1991; Hungria *et al.*, 1991a; Kudou *et al.*, 1991). Nevertheless, an optimization study showed that more complex, non-linear or multistep gradients do improve resolution, as compared to linear gradients (Graham, 1991). Eluents such as acetonitrile and methanol are commonly used in combination with water for gradient chromatography of flavonoid-containing extracts (Graham, 1991). The resolution by several chromatograpic media were compared and included C_4 and C_{18} hydrocarbon-, polyamino-, propylcyano-, and phenyl-substituted columns; their results indicated that a C_{18} hydrocarbon-substituted column resulted in the best resolution (Graham, 1991).

C.1.6. Elicitation and Stress

The first elicitors identified as triggers of isoflavonoid phytoalexin accumulation were pathogenic fungal and bacterial cells (Cruickshank and Perrin, 1961; Lyon and Wood, 1975; Burden and Bailey, 1975). Subsequently, cell wall filtrates of these microbes also proved to act as elicitors (Albersheim and Valent, 1978; Hahn and Albersheim, 1978). Various types of macromolecules were shown to act as 'biotic elicitors', such as arachidonic acid-lipid complexes, eicosapentaneoic acids, protein-lipid-polysaccharide complexes, lipopolysaccharides, glycoproteins and polysaccharrides (Dixon *et al.*, 1983).

C.1.6.1. Biotic Elicitors

The chemical nature of some biotic elicitors has been thoroughly investigated (Albersheim and Valent, 1978; Darvill and Albersheim, 1984). In one particular case, the glycoproteins from *Phytophthora megasperma* lost their elicitor activity on alkaline hydrolysis, but retained it when subjected to treatment with protease or heat (Keen and Legrand, 1980). These results suggested that the branched sugar moieties alone were sufficient for elicitor activity. Other investigations showed that a critical length of nonasaccharide fragments constitutes the minimum requirement for elicitor activity

(Albersheim and Valent, 1978; Hahn and Albersheim, 1978). In other cases, protease treatment did affect the elicitor inducing ability and these were shown to be cell wall degrading enzymes (see endogeneous elicitors, Stekoll and West, 1978). These results suggested that cell surface components can elicit responses in a specific manner. Similar elicitations were obtained with yeast extract (Biggs *et al.*, 1987; Kessmann and Barz, 1987; Bless and Barz, 1988), and ß-1,4-linked glucosamines (chitosan; Hadwiger *et al.*, 1981). Such elicitors allowed the detection of pterocarpan synthase in chick pea cell cultures treated with yeast extract (Bless and Barz, 1988), while chitosan was shown to be an active component of the pea-*Fusarium* interaction (Hadwiger and Beckmann, 1980; Young *et al.*, 1986). These results suggested the effectiveness of ß-glucans as potent elicitors in the biosynthesis of isoflavonoid phytoalexins (Darvill and Albersheim, 1984).

In order to illustrate the mechanism of action of ß-glucan-based elicitors, antibodies raised against cell wall components of *Fusarium solani* and shrimp-shell chitosan were used to investigate the pea-*Fusarium* interaction (Hadwiger *et al.*, 1981). This study revealed that both antisera detected chitosan on the cell surface of the pathogen, in the surrounding medium (pea secretes chitinase enzymes) and inside the cell wall of the host. Such evidence suggests the presence of a specific receptor for the chitosan elicitor on the plant cell surface.

Binding assays conducted with ^{14}C labelled mycolaminaran (a ß-1,3-linked glucan produced by *Phytophthora* species) on soybean extracts fractionated by sucrose gradient, indicated specific binding on the cell membrane fraction of soybean (Yoshikawa *et al.*, 1983). Furthermore, the binding was abolished after treatment with protease or heat, thus suggesting that the plant receptor is a protein or a glycoprotein. Competition binding assays revealed that only branched glucans were able to displace the radiolabeled substrate and, in all cases, the binding correlated positively with the elicitation ability (Schmidt and Ebel, 1987). In addition, investigation of hepta-ß-glucosides as specific receptors in soybean indicated a positive correlation between the degree of binding and elicitor activity

(Cheong and Hahn, 1991). The purification of this hepta-ß-glucoside receptor was accomplished using photoaffinity-^{125}I radiolabeling and glucan affinity chromatography. A 70 kD protein band was shown to be the major form of the heptaglucoside binding protein in detergent-solubilized soybean membranes (Cosio *et al.*, 1992).

Other reports focused on the events following elicitation, especially elicitor binding. It has been shown that elicitors from *Phytophthora parasitica* caused the depolarization of the plasmalemma membranes of *Nicotiana tabacum* root cells (Pellissier *et al.*, 1986). In addition, chitosan was reported to affect membrane permeablity of two leguminous (soybean and common bean) cell suspension cultures (Young *et al.*, 1982). However, it is not known how signal transduction further proceeds inside the cells in these types of interactions (Cosio *et al.*, 1992). Some evidence seems to discard the involvement of cyclic AMP as a potential secondary messenger (Hahn and Grisebach, 1983); however, these mechanisms should proceed similarily to the mechanisms involving cyclic AMP as a secondary messenger (Ebel and Grisebach, 1988). It is, nonetheless, known that the resulting effect will be phytoalexin synthesis.

Elicitation with chitosan has been shown to efficiently trigger phytoalexin accumulation at concentrations ranging from 0.005% to 0.05%, w/v (Pearse and Ride, 1982; Young *et al.*, 1982). Chitosan was also shown to lead to leaching of electrolytes at the highest concentration (Young *et al.*, 1982). Monoterpene indole alkaloids have been reported to accumulate in *Catharanthus roseus* cell suspensions when elicited with chitosan (Eilert *et al.*, 1986).

Yeast extract has also been shown to trigger phytoalexin synthesis in suspension cultures of *Cicer arietinum* at an optimum concentration of 0.25%, w/v (Bless and Barz, 1988).

C.1.6.2. Abiotic Elicitors

Other classes of elicitors have been reported to trigger phytoalexin synthesis. One such class includes the so called 'abiotic elicitors'; mainly heavy metal salts, such as $CuCl_2$ and $HgCl_2$ (Moesta and Grisebach, 1981a; O'Neil *et al.*, 1986; Preisig *et al.*, 1990). Their mode of action can be attributed to a simple disruption of plant cell membranes in a nonspecific manner, or by the release of endogenous elicitors (see later; Dixon *et al.*, 1983). Nonetheless, this mode of action is quite similar to that of ß-glucan-based elicitors; since cell function, via membrane potential disruption, eventually leads to phytoalexin synthesis.

In contrast with the meager knowledge of the mode of action of abiotic elicitors, their role has been well documented. $HgCl_2$ was found to trigger phytoalexin accumulation in soybean (Moesta and Grisebach, 1981a). $CuCl_2$ elicitation at 3 mM enabled the isolation of an *O*-methyltransferase from pea involved in the synthesis of the phytoalexin pisatin (Sweigard *et al.*, 1986). Furthermore, this procedure enabled the enrichment of this enzyme by 370-fold, and thus allowed its purification and characterization (Preisig et al., 1989). More recently, treatment with 5 mM $CuCl_2$ led to the isolation of a new pterocarpan in pea, 2-hydroxypisatin (Kobayashi *et al.*, 1993). Furthermore, $CuCl_2$ elicitation of phytoalexins at low millimolar concentrations, was shown to take place in several *Lupinus albus* cultivars (Shibuya *et al.*, 1992). However, isocratic HPLC analysis of the lupin tissue extracts allowed the quantification of aglycones and monoprenylated derivatives, but not the glucosides or diprenylated compounds (Shibuya *et al.*, 1992). Indeed, such abiotic elicitors have been widely used for the production and identification of new phytoalexins, as well as the purification of the enzymes involved in phytoalexin biosynthesis.

Among other classes of abiotic elicitors are the basic macromolecules (polylysine, histones, autoclaved ribonucleases) and the DNA intercalating agents (9-aminoacridine), as well as treatments with chloroform, fungicides (benomyl), localized freezing, surfactants (Triton X, SDS), and ultraviolet light (Dixon *et al.*, 1983).

C.1.6.3. Endogenous Elicitors

Whereas phytoalexins are synthesized by living cells in response to exogeneous elicitors, it has been shown that endogeneous elicitors also exist in plants (Dixon *et al.*, 1983; Darvill and Albersheim, 1984; Smith and Banks, 1986). In fact, since phytopathogens do infect plants, they must possess enzymatic means to disrupt plant cells in order that invasion takes place. As a result, plant cell wall fragments are released and can act as elicitors. For example, the plant pathogen *Erwina carotovora* secretes a pectin degrading enzyme, α-1,4-D-endopolygalcturonic acid (PGA) lyase (Davis *et al.*, 1982). A such enzyme is isolated from fungal cultures and used to treat soybean, oligogalacturonides are released from the host cell walls. After heat treatment to remove any remaining PGA lyase activity, these oligogalacturonides can be used as elicitors of soybean phytoalexins (Davis *et al.*, 1986).

In the absence of sugar moieties from the phytopathogen the plant cell may, upon invasion, release such endogenous sugar moieties which can then act as modulators of elicitation, thus enabling phytoalexin synthesis. When cell invasion is successful, cell death has been shown to release such elicitors which can signal neighboring cells to synthesize phytoalexins (Dixon *et al.*, 1983).

Accumulation of phytoalexins in plant cells above critical levels can lead to phytotoxicity, i.e. infected cells somehow choose to be destroyed in order to save other healthy uninfected cells. This phenomenon is known as 'necrogenic resistance', and such particular response is called 'hypersensitive reaction' (Dixon *et al.*, 1983; Darvill and Albersheim, 1984; Collinge and Sluzarenko, 1986). Since the accumulation of isoflavonoid phytoalexins is 'classically' post-infectional, a compatible infection by the fungus can lead to the synthesis of a specific isoflavonoid phytoalexin that is not fungitoxic to this species, but would prevent further infection by other pathogens (Smith and Banks, 1986). It is assumed, therefore, that the infected plant copes with the compatible fungus via the hypersensitive reaction described above.

Therefore it can be seen that recognition in host-pathogen interactions is a multistep, multimode, specific process and has proved to be a very efficient one.

C.1.7. Fungitoxic and Bacteriocidal Effects

Isoflavonoid phytoalexins have been shown to inhibit increase in dry weight and radial growth of mycelium, as well as germ tube elongation of fungi (Cruickshank and Perrin, 1961; Cruickshank, 1962; Smith, 1976; Higgins, 1978). At the microscopic level, they alter cytoplasmic streaming, cellular granulation and general organization (Van Etten and Bateman, 1971, Grisebach and Ebel, 1978; Skipp *et al.*, 1977; Bailey and Skipp, 1978). They also cause cell membrane breakdown and the leakage of electrolytes and metabolites (Van Etten and Bateman 1971; Higgins, 1978). Oxygen and sugar uptake by mitochondria is also affected (Slayman and Van Etten, 1974). These manifestations suggest that the fungitoxicity of isoflavonoid phytoalexins is catalysed via membrane exchange processes, which ultimately cause cell death (Smith and Banks, 1986).

However, it has been suggested that resistant microorganisms have isoflavonoid phytoalexin detoxifying enzymes. This could account for the different levels of fungitoxicity a given isoflavonoid phytoalexin may have over different fungal species. For example, prenylated isoflavonoids are metabolized by *Aspergillus flavus* and *Botrytis cinerea* to their corresponding hydrates (Tahara and Ingham, 1987; Tahara *et al.*, 1987c). These studies suggest that some fungal species may have hydratases specific to prenyl groups, which enable them to cope with prenylated isoflavonoid phytoalexin fungitoxicity. In addition, methylated compounds such as phaseollidin are detoxified by *Fusarium*, the bean pathogen (Smith *et al.*, 1980).

Although considered bacteriostatic or bacteriocidal, the effect of isoflavonoid phytoalexin on bacteria has been much less documented as compared with pathogenic fungi (Smith and Banks, 1986). While the term 'bacteriocidal' refers to capability of killing bacteria, 'bacteriostatic' defines the ability to inhibit bacterial growth without killing them

(Brock *et al.*, 1984). It should be noted, however, that the distinction between both terms is often arbitrary, since an agent can be bacteriocidal at high concentration while it may only be bacteriostatic at a lower one (Brock *et al.*, 1984). Nonetheless, their antibacterial activity has been shown to affect membrane processes. In fact, glycinol is suspected of intercalating with bacterial membranes, thus impairing their semipermeable function (Weinstein and Albersheim, 1983).

A tentative generalization has been made that Gram-negative bacteria are usually less sensitive to flavonoid phytoalexins than Gram-positive ones (Gnanamanickam and Mansfield 1981). However, this is not corroborated by the data obtained with glycinol, where the growth of Gram-negative strains was more inhibited than Gram-positive strains (Weinstein *et al.*, 1981). In addition, no correlation could be made between growth inhibition and pathogenicity involving glycinol. Nonetheless, this tentative generalization is reflected by the increased growth rate observed in some *Rhizobium* species (Gram negative) when exposed to *nod* gene inducers (flavonoids) at 1 to 5 μM concentrations in minimal liquid media, although most of the compounds tested were not phytoalexins (D'Arcy-Lamenta and Jay, 1987; Hartwig *et al.*, 1991).

Several assays have been used to assess the effects that isoflavonoid phytoalexins may have on bacterial growth. Qualitative assays involve the use of solvent-solubilized isoflavonoid phytoalexin-impregnated disks that are deposited on bacteria-seeded agar (Skipp *et al.*, 1977; Bailey and Skipp, 1978; Gnanamackinan and Mansfield, 1981). The appearance of growth inhibition rings around the disks is used to assess the inhibitory activity of a given isoflavonoid phytoalexin, in a manner similar to the standard assays for antibiotics. In addition, preliminary screening to assay for growth inhibition of fungi can be accomplished by spraying the fungal spores suspended in the culture medium onto developed TLC chromatograms of a given plant extract (Homans and Fuchs, 1970). Alternatively, known amounts of isoflavonoid phytoalexins may be added to the bacterial cultures (Pankhurst and Biggs, 1980; Parniske *et al.*, 1991a). The bacterial growth is

monitored (OD_{600}) at regular time intervals, as well as the protein content and viable counts. The latter methodology enables a more accurate quantitative calibration of the effect of a given compound on a particular bacterial strain. In these studies, various phytoalexins were assayed at concentrations between 10 µM and 1 mM (Pankhurst and Biggs, 1980), 90 µM and 270 µM in liquid cultures (Parniske *et al.*, 1991a), or at 200 µg per disk with the growth inhibition disk assay (Weinstein and Albersheim, 1983).

1.8. The isoflavonoids of *Lupinus albus*

The phytochemistry of *Lupinus* isoflavonoids has been extensively studied by Dr S. Tahara, and resulted in the elucidation of >30 structural types of isoflavonoids. Genistein, a 5,7,4'-trihydroxyisoflavone, was first isolated at the beginning of this century from Dyer's broom, *Genista tinctoria* (Figure 2; Perkin and Newburry, 1899). It was shown to be present in many *Lupinus* species and to be the precursor of all other lupin 5-hydroxy-isoflavonoids (Schröeder *et al.*, 1979; Tahara *et al.*, 1984a; Hagmann and Grisebach, 1984). 2'-Hydroxylation of genistein yields 2'-hydroxygenistein (Ingham, 1972) and further cyclization of this hydroxyl with C-2 yields lupinalbin A, a simple coumaronochromone present in *L. albus* (Figure 2; Tahara *et al.*,1985a).

The first prenylated isoflavonoid to be isolated was luteone from immature fruit of *L. luteus* (Fukui *et al.*, 1973), whose structure was shown to be a 6-prenyl derivative of 2'-hydroxygenistein (Figure 2; Harborne *et al.*, 1976). Its constitutive synthesis was shown to take place in a number of *Lupinus* spp. and its proven antifungal activity qualified it as a pre-infectional agent (Harborne *et al.*, 1976). Another unidentified compound in the latter study also exhibited such properties (Harborne *et al.*, 1976), and its structure was later determined to be the 3'-prenyl derivative of 2'-hydroxygenistein, or licoisoflavone A (Figure 2; Tahara *et al.*, 1984a).

Other prenylated isoflavonoids were also isolated from *L. albus* and their structures were elucidated by spectroscopic methods. Wighteone and lupiwighteone were determined

to be the 6- and 8-prenyl derivatives of genistein, respectively (Figure 2; Tahara *et al.*, 1984a). A 3'-prenyl derivative of genistein was also isolated from *L. albus* and named isowighteone (Figure 2; Tahara, personal communication). However, no 8-prenyl derivative of 2'-hydroxygenistein (2,3-dehydrokievitone) was found in *L. albus*, although it was found in *L. luteus* (Tahara *et al.*, 1985a; Shibuya *et al.*, 1992). In addition, the 3',6-diprenyl derivatives of both genistein and 2'-hydroxygenistein were isolated from *L. albus* and named lupalbigenin and 2'-hydroxylupalbigenin, respectively (Figure 2; Ingham *et al.*, 1983; Tahara *et al.*, 1989b). The 7-*O*-glucosides of both aglycones were also found in *L. albus* (Figure 2), as well as other glucosides (7 and 4') and their malonyl esters (Shibuya *et al.*, 1991). Malonylglucosides have also been found in soybean (Kudou *et al.*, 1991). More recently, 7-*O*-glucosides of prenylated isoflavones have been isolated and characterized from white lupin (Shibuya *et al.*, 1991).

Several coumaronochromones that are substituted with prenyl, furano and pyrano groups also occur as minor constituents of *L. albus* (Figure 2; Ingham *et al.*, 1983; Tahara *et al.*, 1984a; 1985a; 1989b; 1990). In contrast with soybean which produces prenylated pterocarpans post-infectionally (Weinstein *et al.*, 1981) as well as minor pterocarpanoids (Lyne *et al.*, 1976; Lyne and Mulheirn, 1978), lupin roots are capable of constitutively expressing the synthesis of prenylated coumaronochromones (Tahara *et al.*, 1984a). In addition, several methyl ethers of luteone (Tahara *et al.*, 1987d) and of licosioflavone A (Tahara *et al.*, 1987a) occur as minor constituents of *L. luteus*. The phytochemistry of isoflavone formation has also been studied in suspension cultures of *Lupinus* species cultures (Berlin *et al.*, 1991a; 1991b; Hallard *et al.*, 1991).

Furthermore, the fungal metabolism of luteone (Tahara *et al.*, 1984b), licoisoflavone A (Tahara *et al.*, 1985b), 2,3-dehydrokievitone (Tahara *et al.*, 1987a), and edunol (Tahara and Ingham, 1987), as well as the stereochemistry of their microbial derivatives (Tahara *et al.*, 1987b) have been extensively studied and allowed identification of an epoxy intermediate common to these fungal biotransformations (Tahara *et al.*, 1989a). Luteone

was recently shown to be metabolized in a rat liver homogenate yielding dihydrofuranosides (Sugawara *et al.*, 1991), with structures similar to those obtained with fungal biotransformation (Tahara *et al.*, 1989a).

C.2. *RHIZOBIUM*-LEGUME INTERACTIONS

C.2.1. Classification and Distribution of *Rhizobium* Species

It is interesting to recall the 'pathogenic nature' that was originally assigned to nodules in the XIXth century. The nodules occurring on the root systems of various Leguminosae were believed to be insect galls or other pathogenic manifestations (Brock *et al.*, 1984). This may have contributed to the classification of both *Rhizobium* and *Agrobacterium* (common plant pathogens) species into the Rhizobia family, as well as other nitrogen fixation genera which nodulate non-legume hosts (Smith and Douglas, 1987). However, the majority of *Rhizobium* species are currently classified into two separate genera, *Bradyrhizobium* and *Rhizobium* (Bergey's Manual, 1984). This taxonomy is based on some distinguishing features including their growth rates (slow or fast, repectively), the location of their symbiosis-related genes (on the chromosome or on a megaplasmid, repectively), bacterial morphology inside nodules (swollen rods vs pleiomorphic) and the physiology of symbiotic nitrogen fixation (ureide vs amide) to mention a few (Smith and Douglas, 1987).

Rhizobium species are classified on the basis of their ability to nodulate legumes. Species classification is related to the host(s) each strain is able to nodulate, i.e. symbiosis of one species is restricted to one (or several) hosts, in a species-specific manner as shown in Table 1 (Long, 1989).

In 1886, the German scientist Hermann Hellriegel demonstrated the ability of legumes to grow in nitrogen deficient soils, whereas non-legumes could not. Yet, legumes were unable to form nodules in sterilized soils; but when inoculated with crushed nodules, legumes grown under sterile conditions did nodulate (Brock *et al.*, 1984). At this point

two facts were established; (a) bacteria are required for nodulation to occur, and (b) symbiosis enables the infected plant to substain growth in a nitrogen-poor environment. Following these findings, much attention was directed to the agricultural benefits of *Rhizobium*-legume association, as to the effects of crop rotation on crop yields and to sustain high nitrogen levels in soils. Indeed, research had to await new techniques, especially those related to molecular biology, in order to reveal the basis of this symbiotic interaction.

Rhizobium species are Gram-negative motile rods, 0.5 to 0.9 mm wide and 1.2 to 1.3 mm long, that live freely in soils (Smith and Douglas, 1987). Their abundance in the rhizosphere increases substantially in the vicinity of their host (Taylor and Beringer, 1981), and they are not usually found in soils that have never borne legumes (Smith and Douglas, 1987). Experiments showed that when mixed *Rhizobium* populations (motile and non-motile strains) were used to inoculate a host plant, only motile strains were found in the resulting nodules (Ames and Bergman, 1981; Cétano-Annolès *et al.*, 1988b). These bacteria are chemotactic towards localized regions of the legume's root and can migrate in a petri dish at 2 cm/day (Gulash *et al.*, 1984). It was also demonstrated that flavonoid molecules, the inducers of *nod*-genes (see later) were responsible for this positive chemotaxis (Caétano-Annollès *et al.*, 1988a). Furthermore, mutations affecting the chemotaxis toward these molecules were shown to physically map on the megaplasmid. Indeed, when a large 290 Kb deletion mutant (ca. *nif-nod*) of *R. meliloti* was assayed, it lost positive chemotaxis towards flavonoids, but not towards sugars or amino acids (Caétano-Annollès *et al.*, 1988a). These results led to suggest "a dual pathway for *Rhizobium* chemotaxis" (Bergman *et al.*, 1988). In addition, chemotaxis of wild type strains was negative toward other flavonoid molecules, the inhibitors of *nod* genes (see later; Bergman *et al.*, 1988). Therefore, symbiotic genes of *Rhizobium* species enable chemotaxis towards a compatible host plant and this phenomenon is modulated by the

flavonoids that are exuded in the rhizosphere (Gulash *et al.*, 1984; Parke *et al.*, 1985; Bergman *et al.*, 1988; Caétano-Annollès *et al.*, 1988a).

C.2.2. The Infection Process

The different stages of *Rhizobium* infection have been well characterized (for reviews see: Long, 1989; Nap and Bisseling, 1990; Hirsch, 1992), and they will be briefly discussed here, with phenotypical notation given in parentheses. *Rhizobium* will 'swim' towards its host and attach to the root hair cells, causing root hair deformation (*Had*+) (Vincent, 1980; Vasse and Truchet, 1985). This is followed by a typical root hair curling into a sheperd's crock (*Hac*+), which consists of a 180° twist of the root hair. As this happens, cortical cells under the epidermis start to divide (*Crd*+) via dedifferentiation into a meristem (Libbenga and Harkes, 1973; Dudley *et al.*, 1987). An infection thread, probably derived from the root hair's cell wall (Bradley *et al.*, 1988), enables the bacterium to penetrate inside the host cell. The ramification of the infection thread helps to direct the bacteria towards the underlying dividing cortical cells, causing nodule initiation (*Nol*+) (Scheres *et al.*, 1990). These processes (*Hac*+, *Crd*+ and *Nol*+) require entire bacterial cells to take place, as opposed to root hair deformation (*Had*+) which can be initiated only in presence of the bacterial supernatant (Roche *et al.*, 1991). Upon destination, the symbionts lose their cell wall and become enclosed in a peribacteroid membrane, derived from the host cell membrane (Verma *et al.*, 1978). This nodule structure affords a microaerophilic environment in which the bacteria will be able to fix nitrogen. Metabolite exchange of ammonia and carbohydrates provides a benefical interaction for both host and symbiont, thus meeting the requirements of a symbiotic interaction (Smith and Douglas, 1987).

C.2.3. The Symbiotic Genes

It was in 1981 that genes controlling early and late functions in symbiosis were found to be located on a megaplasmid (pSym) in *R. meliloti* with a size of 1500 Kb (Rosenberg *et al.*, 1981). This study demonstrated that all the 27 strains of *R. meliloti*, from different geographical areas, are harboring symbiotic genes on their megaplasmid in the absence of which they lose the ability to nodulate their host (Marvell *et al.*, 1987). *Rhizobium* megaplasmids range from 200 Kb in *R. leguminosarum* to 1500 Kb in *R. meliloti* (Long, 1989). The availability of a new cosmid vector (Ditta *et al.*, 1980), compatible with Gram-negative bacteria, enabled the construction of a gene bank from *R. meliloti* pSym (Friedman *et al.*, 1982; Djordjevick *et al.*, 1983), and of translational *LacZ* fusions in order to study *nod* gene expression (Mulligan and Long, 1985). In the meantime, several genes of the megaplasmid have been mapped and sequenced, while the characterization of several nodulation mutants has enabled the establishment of different phenotypical classes.

Some classes of mutations were found to be required for the early steps of infection (*Had*, *Hac*, *CrD*, and *Nol*), while others do affect nodule function. The former are referred to as 'early nodulation genes', and the latter as 'late nodulation genes'. Both early and late nodulation genes are further sub-divided into several categories which physically map on the megaplasmid, but are distinctly located (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* and *CrD* phenotypes. Other genes, including those which produce exopolysaccharides (*Exo* genes), lipopolysaccharides (*Lps* genes; Djordevic *et al.*, 1987) and cyclic glucans (*Ndv* genes; Dylan *et al.*, 1986) are thought to be involved in host cell penetration and formation of the infection thread (Gray and Rolfe, 1990). While *nod* genes do induce a host response, other early genes are considered to act as 'avoidance determinants' of the plant defense systems (Nap and Bisseling, 1990).

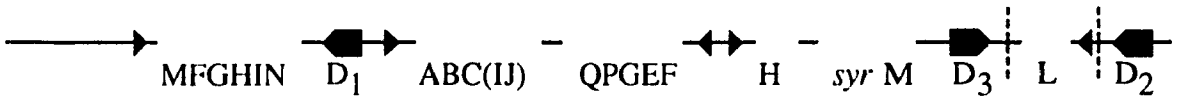
The late nodulation genes are not involved in host specificity, but code for enzymes involved in nodule functioning, such as nitrogen fixation enzymes (*ntr* and *fix*). Those having sequences homologous to nitrogen fixation enzymes of *Kleibsella* are called *ntr* genes (Drummond *et al.*, 1986; Buikema *et al.*, 1987), while those restricted to symbiotic nitrogen fixation are known as *nif* genes (Long, 1989). Genes coding for enzymes involved in the biosynthesis of the heme moiety of leghemoglobin also belong to the late nodulation genes (Leong *et al.*, 1982; Fuller *et al.*, 1983). Interestingly, leghemoglobin is considered to be the only true symbiotic protein known, since its functioning requires the genomes of both host and symbiont (Nap and Bisseling, 1990).

C.2.4. *Nod* Genes: Structure, Induction and Inhibition

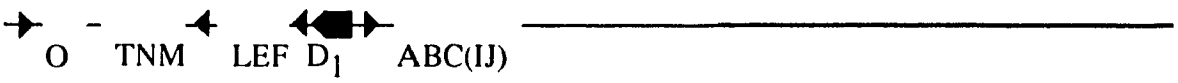
In order to discuss the *nod* genes in more detail, I shall use *R. meliloti* as an example, since most of the pioneering work has been carried out with this species (Horvath *et al.*, 1986). The *nod* genes of *R. meliloti* are arranged in six operons, each of which is preceeded by a Nod box (Figure 5; Hirsch, 1992). The Nod box is a 47-bp strictly conserved region in the extended promotor region of each *nod* operon, as revealed by sequence homology studies (Rostas *et al.*, 1986). In addition, this conserved sequence was also found in several other *Rhizobium* species. The *Nod* box is, therefore, a common promotor sequence for all *Rhizobium nod* genes. However, the constitutively expressed *nodD* gene lies upstream from the Nod box of the *nod ABC(IJ)* operon, and is transcribed in opposite directions (Mulligan and Long, 1985).

The first operon to be characterized contains the *nodABCIJ* genes, these genes are called the 'common *nod* genes', since their sequence is highly conserved in all *Rhizobium* species (Friedman *et al.*, 1982), and because they functionally complement similar genes in other *Rhizobium* species (Long *et al.*, 1982). The other operons are referred to as the 'host specific genes', since they are not present in all *Rhizobium* species (Long, 1989). They are

Nod-genes of *Rhizobium meliloti*



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



Nod-genes of *Bradyrhizobium japonicum*

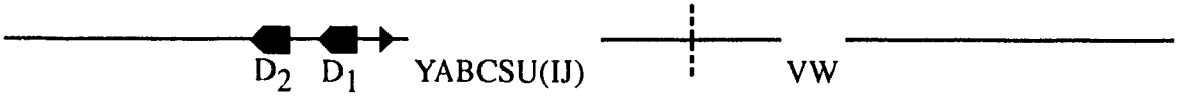


Figure 5: Physical map of *Rhizobium nod*-genes. With the exception of *syr* (symbiotic regulator), all letters refer to *nod*-genes. The black triangles refer to *nod*-boxes, while the dashed vertical lines designate large interruptions in the map. The genes are not drawn to scale. After Hirsch, 1992.

grouped as follows: *nodFEGPQ*, *nodMFGHIN*, *nodM*, *nodH*, and *nodL* (Figure 5; Debellé *et al.*, 1986; Hirsch, 1992).

In order to monitor the expression of *nodD* and *nodABC(IJ)*, Mulligan and Long (1985) used *R. meliloti* strains transformed with translational *lacZ* fusions (and Tn5 insertions; Djordjevic *et al.*, 1985), with an assay for ß-galactosidase activity in the presence (or absence) of alfalfa exudates. Their assays revealed that *nodD*, as well as the alfalfa exudate, were required for *nodC* expression, since the pRmM57 construct (*nodABC(IJ)*::*LacZ*, *nodD*::*Tn5*) did not yield any ß-galactosidase activity as compared with constructs harboring functional *nodD* (Mulligan and Long, 1985). These results suggested a role for *nodD* in the regulatory 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).

The inducer molecule responsible of *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 identified as luteolin, a plant flavone (3',4',5,7-tetrahydroxyflavone), based on ultraviolet-visible absorption spectra, proton nuclear magnetic resonnance, and mass spectrometric analyses (Peters *et al.*, 1986). In addition, synthetic luteolin also exhibited inducing activity and spectral characteristics similar to those of the natural alfalfa inducer.

Similar investigations conducted on the exudates of various leguminous hosts and their specific *Rhizobium* species (which were transformed using similar translational *lacZ* fusions), led to the discovery of *nod*-gene inducers in other symbiotic systems (Table 1). The various examples shown demonstrate the wide diversity of flavonoids which can act as inducers of *nod*-genes and thus contribute to host specificity. Indeed, these examples (Table 1) show that flavones, isoflavones, flavonols, flavanones, dihydroflavonols and anthocyanidins can all act as inducers.

In addition, several inducers may be exuded naturally from a host root, and each of which may have a different inducing potential. This phenomenon has been well illustrated by testing three *Rhizobium* species 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 induced a wide range of ß-galactosidase levels. In addition, these studies revealed a requirement for a 7-hydroxylation (flavone numbering system) and, to a lesser extent, for a 4'-OH group. The requirement for a 7-OH was also reported in an earlier work on *R. trifolii* (Redmond *et al.*, 1986).

Furthermore, it was demonstrated that clover seedlings grown on X-Gal-containing agar plates and inoculated with *R. trifolii* harboring *nodC::lacZ* fusions, yielded blue and white zones along the root system (Redmond *et al.*, 1986). These results suggested two points. First, there might be a differential pattern for the release of flavonoid inducer along the root system of the plant host, which would enable the latter to control the extent of nodulation by differential synthesis and release of the flavonoid inducer(s). Second, the host may have the means of antagonising the effect of the inducer on the microsymbiont, thus allowing to control the extent of nodulation. It is interesting to note that white lupin differentially synthesizes isoflavones along its hypocotyl (Ferrer *et al.*, 1990; Ferrer *et al.*, 1992) such that it may be able to control nodulation along the root system as well, although emphasis in the latter report was placed on the role of isoflavonoids in cell wall peroxidase activity.

Molecules which antagonize the effect of *nod* genes-inducer have been isolated in other symbiotic systems (Table 1). Using fully induced *R. leguminosarum* incubated with commercially available flavonoids, a reduction in the level of induced activity was observed with two isoflavones (genistein and daidzein) and one flavonol (kaempferol; Firmin *et al.*, 1986). Similar experiments revealed such antagonistic activity in *R. meliloti* (Peters and Long, 1988) where apigenin, naringenin and eriodictyol have been shown to reduce the inducing activity of luteolin. In analogy with inducers, the inhibitors of *nod* genes were

shown to produce negative chemotaxis. The evidence that *nod* gene inhibitors were also present in the exudates of *R. meliloti* (Peters and Long, 1988), further substantiates the involvment of flavonoids in the control of nodulation.

C.2.5. *Nod* genes: Regulation of Expression

The *nodD* amino-terminal sequences are highly conserved among different *Rhizobium* species, and they show homologies with the proposed DNA binding sites contained in *E. coli* regulatory genes (Burn *et al.*, 1987). It was later shown that gel retardation of promotor regions of *nod* genes, with the addition of *nodD* protein, indeed takes place (Hong *et al.*, 1987). Another line of evidence showed that the *nodD* product binding to the *nodA* promotor is independent of the presence of inducing flavonoid (Fisher *et al.*, 1988). These features suggest that the *nodD* protein functions as a DNA binding protein, and thus acts as a regulator of *nod* genes expression.

The construction of chimeric *nodD* genes from *Rhizobium* MPIK3030 (a wide host-range strain) and *R. meliloti* allowed to demonstrate that altered *nodD* sequences affect bacterial response to different plant signals. The results of these studies indicated that the carboxy-terminal region of the *nodD* protein may be responsible for the interaction with the plant factor (Horvath *et al.*, 1987). More recently, chimeric *nodD* from different *Rhizobium* species have enabled to alter the flavonoid specificity of the resulting proteins (Spaink *et al.*, 1989). These results confirmed that the carboxy-terminal is indeed involved in the specificity of activation by flavonoids.

Furthermore, immunocytochemical localization studies revealed that the *nodD* protein is located in the cytoplasmic membrane of *R. leguminosarum* in the absence of the host exudate (Schlaman *et al.*, 1989). Other reports demonstrated that naringenin (the inducer of *R. leguminosarum bv vicia*, exuded naturally from its host) accumulates on the cytoplasmic membrane of *R. leguminosarum bv viciae* (Recourt *et al.*, 1989). Since inducible *nod* genes are not required for naringenin accumulation on the cell surface

(Recourt *et al.*, 1989), and because of the membrane localization of the *nodD* protein, it is assumed that naringenin binds to this protein (VanBrussel *et al.*, 1990). The acid labile protons formed may cause a disruption in the protein charge, leading to conformational changes on its DNA binding domain, thus enabling expression of the inducible *nod* genes.

Therefore, the *nodD* protein may contain three functional domains: a DNA binding one on its amino-terminal region, which can bind to the *Nod* box (in the absence of the inducer); a putative hydrophobic, 'middle' domain, which enables the protein to be located on the cell membrane, and a flavonoid-binding domain at the carboxy-terminal region. The latter contributes to host specificity, since it can react to both inducer(s) or inhibitor(s).

In addition to the characteristics of *nodD*, which confer some of the host specificity, other *nodD* genes were discovered in the *R. meliloti* symplasmid (Honma and Ausubel, 1987). These genes have common *nod*-promoter activation abilities but diverge in their recognition of different flavonoids, although the two new *nodD* alleles were not constitutively expressed (Gyorgypal *et al.*, 1988).

C.2.6. *Nod* Genes: Products and the *Ini* Phenotype

When all *nod* genes are expressed from a wild type *R. meliloti*, due to the presence of the flavone inducer, their expressed proteins synthesize and secrete a bacterial factor that was called NodRm1 (Lerouge *et al.*, 1990). This factor has been purified by gel permeation, ion exchange and 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*-($C_{16:2}$)acyl-tri-*N*-ß-1,4-D-glucosamine tetrasaccharide. The purified NodRm1 factor was active at nanomolar concentrations in root hair deformation on alfalfa, but not on vetch (an heterologous host; Lerouge *et al.*, 1990). Other Nod factors have also been isolated from *R. l. bv. viceae* (Spaink *et al.*, 1991; Shultze *et al.*, 1992). They have similar structure, but also show significant differences (Okker *et al.*, 1992).

R. meliloti nodP and *nodQ* have DNA sequence homologies with the *cysDNC* locus of *E. coli*, which code for proteins responsible for adenosine 5'-phosphosulfate (APS) synthesis, the first activation step of inorganic sulfate (Schwedock and Long, 1989). Such activities were demonstrated for *nodP* and *nodQ*, both *in vitro* and *in vivo* (Schwedock and Long, 1990). In addition, *nodH* is homologous to a sulfotransferase (Roche *et al.*, 1991). Indeed 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 on alfalfa, but causes root hair curling on the heterologous host, vetch, when applied at concentrations of 10^{-8} to 10^{-9} M (Roche *et al.*, 1991). This example well illustrates how the host range specificity can be altered when host-specific *nod* genes are mutated.

The functions of most of other *nod* proteins have been suggested based on homologies with other known proteins, and all suggest involvment in the biosynthesis of the lipo-oligosaccharide factor (Jacobs *et al.*, 1985; John *et al.*, 1988; Downie *et al.*, 1991; Baev *et al.*, 1991; Spaink *et al.*, 1991; John *et al.*, 1993). Host-specific *nod* genes thus contribute to specify structural features of *nod* factors.

Nod factor's side chain length (2 nm) are approximately half the depth of a lipid bilayer which suggests a membrane localization, and is supported by its amphoteric nature (Hirsch, 1992). This would imply that, at least, some of the *nod* factor remains embedded in the bacterial membrane, although most of it is excreted. The specific oligosaccharides of this factor (sulfated or not) may stick out of the membrane, and thus contribute to specific binding on the host root hair cells. We have seen that such sugar moieties can specifically bind to plant receptors in host-pathogen elicitation mechanisms (Yoshikawa *et al.*, 1983).

A 'lectin binding theory' was first proposed by Bohlool and Schmith (1974) to explain *Rhizobium* attachment to the host cell wall. Several lines of evidences corroborate with this suggestion (Halverson and Stacey, 1986). Anti-lectin antibodies have been shown to bind to regions which correlate with sites of infection in pea roots (Diaz *et al.*, 1986). However,

it was demonstrated that bacterial fibrils synthesized from chromosome-borne genes were responsible for attachment on the root hair cells (Smit *et al.*, 1986), which implies that specific pSym genes are not required for attachment. Nonetheless, increased *nod* gene expression did occur upon attachment of *R. leguminosarum* to *Vicia sativa* roots (VanBrussel *et al.*, 1990). This phenomenon was called the 'Increased nodulation induction' phenotype, (*Ini*). It is hypothesized that the *nod* factor, 'via specific binding to lectins' would then enable bacteria to be engulfed (as the root curls) much like coated pits are involved in active endocytosis. This hypothesis has been put foreward to explain bacterial penetration (VanBrussels *et al.*, 1990; Hirsch, 1992). Therefore, attachment is accomplished via fibrimae, whereas initial penetration (or root hair curling) is accomplished via specific binding of the *nod* factor.

The *Ini* phenotype, and its putative function (i.e. for active endocytosis) have been substantiated by additional evidence. The Dénarié group have shown in 1991 that the NodRm1 caused Had (deformation) at 10^{-11} M, but that 10^{-7} M was required for Hac and CrD to take place. So, more *nod* factor is required to cause cortical cell division and root hair curling, both of which are prerequesites for proper bacterial penetration. This corroborates with the *Ini* proposal and implies that more *nod* factor is needed for host penetration by the bacterium.

An additional feature is needed for the *Ini* proposal to stand criticism; that is, an additional release of the inducer is required for the *Ini* phenotype to take place. Several cases reported in the literature support this fact. One recent report investigated the alteration of flavonoid pools in *Lotus pedunculatus* roots upon infection with *Rhizobium loti* (Cooper and Rao, 1992). They observed that qualitative changes in flavonoid composition take place in the nodules as compared with roots of both inoculated and uninoculated plants. No further interpretation is possible from such experiment, since this study was accomplished using paper chromatography, which does not allow to discriminate between individual compounds of the flavonoid groups. In addition, activation of flavonoid

biosynthesis in *Vicia sativa* roots takes place after inoculation with *Rhizobium leguminosarum bv. viciae* (Recourt *et al.*, 1992). In this study, they monitored PAL enzyme activity, levels of CHS mRNA expression, and the activity of methyltransferases involved in the formation of eryodictyol, the inducer, in the root system. Their results showed an increase in enzyme activities and mRNA expression in inoculated systems as compared with uninoculated ones.

Other reports on soybean are more interesting because data are avalaible for both HPLC analysis of flavonoids and mRNA expression of PAL and CHS. One study has shown that *Rhizobium* attachment causes an increase in specific PAL and CHS gene expression upon infection (Eastabrook and Sengupta-Gopolan, 1991), thus implying increased flavonoid synthesis (as is the case with isoflavonoid phytoalexin accumulation). Indeed, glyceollin I (an isoflavonoid phytoalexin) has been shown to accumulate in root hairs (but not exuded) after infection, although no mention was made of daidzein (an inducer) accumulation (Schmidt *et al.*, 1992). However, it would be reasonable to assume the presence of daidzien since it is the precursor of glyceollin I. To further substantiate this view, it was previously shown that addition of the inducer to the rhizosphere increased nodulation (Kapulnick *et al.*, 1987).

The lectin binding theory, therefore, describes very accurately the initial penetration in a specific manner, while the *lni* phenotype along with other evidences support this view. In a case where too many *Rhizobium* cells may attach to an host, penetration can be arrested by the sole shut-down of an inducer's synthesis, or inversely an increase in an inhibitor's synthesis, or simply via phytoalexin synthesis and accumulation.

C.2.7. Host Nodules and Secondary Roots

The ultimate morphological effect of *Rhizobium* infection is the formation of nodules which are considered to be plant organs (Nap and Bisseling, 1990). Two types of nodules are observed in legumes, 'determinate' and 'indeterminate', although each type is host-

dependent, but not symbiont-dependent. The main difference between these two nodule types is functional. Determinate nodules have distinct functional stages, some of which are in the formation stage, other fix nitrogen or senesce (Calvert *et al.*, 1984); whereas indeterminate nodules maintain all stages of nodule functions within the same structure. Indeterminate nodules typically contain a growing apex, followed by a nitrogen prefixing zone, a nitrogen fixing zone and finally a senescent zone (Dudley *et al.*, 1987; Hirsch, 1992).

It has been suggested that nodule morphology resembles that of secondary (lateral) roots in many respects. This comparison is more evident with indeterminate than determinate nodules. Lateral roots are regularly positioned along the primary root, whereas nodules form at zones with growing root hair or at the site of lateral root formation (Libbenga and Bogers, 1974). In addition, if the primary root tip is excised, both lateral root formation and nodulation are enhanced, probably due to redistribution of apical dominance (Cétano-Anollès *et al.*, 1991). Nodule formation also decreases the number of lateral roots formed, at least in clover (Libbenga and Harkes, 1973). More specifically, when legumes are infected with mutant strains unable to form infection threads, they lead to empty nodules (Wolfe and Werner, 1990) whose morphology closely ressembles that of lateral roots (Dudley *et al.*, 1987). In addition, legume nodules are highly organized, as opposed to tumors resulting from infection by *Agrobacterium* (Hirsch, 1992).

Determinate nodules have been shown to accumulate glyceollin I in soybean (Karr *et al.*, 1992), which are also formed as phytoalexins after elicitation of soybean cultures and seedlings (Zärhinger *et al.*, 1979; Zärhinger *et al.*, 1981; Schmelzer *et al.*, 1984; Hanh *et al.*, 1985). More specifically glyceollin I is bacteriostatic towards *Bradyrhizobium japonicum* (Parniske *et al.*, 1991a). In one study, glyceollin I accumulation was monitored in two symbiotic systems, a compatible strain capable of nitrogen fixation in the soybean cultivar used, and an incompatible strain that yielded non-functional nodules (Karr *et al.*, 1992). Glyceollin I accumulation in the nodules formed by a compatible strain reached 60

µg/g f.wt. while it only reached 10 µg/g f.wt. in the adjacent tissues. On the other hand, results obtained with the incompatible strain yielded 400 µg/g f.wt. and 50 µg/g f.wt. glyceollin I, respectively. Whereas the accumulation of phytoalexin coincides with the decrease in nitrogen fixation activity (e.g. in the compatible system), glyceollin I accumulates in adjacent regions but at much lower levels. It seems likely that the host eventually kills the symbiont by accumulating bacteriocidal levels of the phytoalexins when symbiosis becomes less beneficial. In the incompatible situation, much higher levels are reached, and indeed, symbiosis is not beneficial in this case. Another report on soybean showed that accumulation of glyceollin I in nodules reached 6 to 8 µg/g f.wt. (ca: 2.8 nmol/mg f.wt.) when inoculated with a *nif*⁻ strain as compared with the controls (Parniske *et al.*, 1991b). In this case, however, accumulation was faster and higher, and yielded symptoms of hypersensitive reaction. These two experiments were conducted on different soybean cultivars, so that it may seem that the level of accumulation was cultivar-dependent. Indeed, this was found to be the case in *Vicia faba* (Wolfe and Werner, 1990). Therefore, *Rhizobium* leads to phytoalexin-like patterns of accumulation in nodules, and the levels reached are host-dependent.

Other investigations indicated that induction of PAL and CHS gene expression correlated with the abortion of infection thread (those not yielding nodules; Lullien *et al.*, 1987). Therefore, this demonstrates an additional aspect of symbiosis in which flavonoids (especially phytoalexins in the present case) may exert some control over the extent of nodulation.

C.2.8. Host Control over Nodulation.

The host plant's control of the extent of nodulation has been well documented in the literature. It has been observed that when 10 seedlings of alfalfa (ca. 80,000 root hairs) are inoculated with *Rhizobium*, 25% of the root hairs will deform, 52 infection threads are formed, and yet only 27 nodules result on the ten seedlings (Wood and Newcomb, 1989).

Other investigations revealed a positive correlation between the amount of flavonoids and the amount of nodules (Cho and Harper, 1991). Nitrates present in the rhizosphere also lead to an increase in isoflavonoid synthesis (Cho and Harper, 1991). These control features have been further illustrated by the split root system developed for soybean (Kosslak and Bohool, 1984). In this system, the sterile root systems of soybean seedlings are split into two growth medium-containing chambers. *Bradyrhizobium* inoculation of one chamber resulted in the formation of nodules ten days later. Re-inoculation with similar bacterial titers of the sterile chamber did not yield any more nodules. These results indicate that control must have, somehow, stopped infection in the second chamber and that the host response was systemic.

It has been suggested that soybean may control nodulation by a feedback regulation mechanism (Calvert *et al.*, 1984; Caétano-Anollès and Bauer, 1988). In addition, both the root apical meristem and mature nodules elicit feedback regulation on nodule formation (Caétano-Anollès *et al.*, 1991). Therefore, nodulation is a controlled process that is at least partly modulated and regulated by flavonoids.

Inducers of *nod* genes are released from alfalfa's root, i.e. exuded, at a rate of 1 to 3 pmol/plant/hr (Maxwell and Phillips, 1990). Quantification of alfalfa 'seed effusates' are reported at rates of 70 pmol/plant/hr (Hartwig and Phillips, 1991). That is to say, inducers are contained at much higher levels in the seed coat than they are exuded from the seedling root. In fact they constitute a pre-existing pool in seed coats ready to be secreted. *Vicia faba* has also been shown to accumulate inducers in mature pods (Tomas-Lorente *et al.*, 1990). It maybe that imbibition by the seed releases more inducer, so that it may attract more compatible bacteria. However, when they get closer to the host plant, the release is much lower and thus only a few can attach successfully. The host may further vary its flavonoid pools according to the infection it can withstand, along with the feedback mechanisms already discussed. Phytoalexins are used to control post-infectional events, whereas simple flavonoids are used to control pre-infectional events.

Flavonoids of the Leguminosae play a central role in plant interactions with *Rhizobium*. Flavonoids are the determinants of host specificity in three respects. First, inducers will direct the bacterial cell towards definite sites on the host root system. Second, they will induce *nod* gene expression in the bacteria and favor their growth. Third, they will enable host-specific binding on the root hair cells because of the *nod* factor they produce. In addition, increase in inducer synthesis (the *Ini* phenotype) in the root upon attachment will also enable proper penetration. The differential synthesis of inducers and inhibitors along the root system, or the preferential release of weaker inducers over stronger ones, will enable the host to control the number of infections, both spatially and temporally. In addition, a shift of flavonoid synthesis towards the accumulation of phytoalexins after penetration, will enable further limitation in the level of infection, or will lead to senescence in parts of the nodules (indeterminate), or entire nodules (determinate).

D. MATERIALS AND METHODS

All experiments described in this Section deal with two distinct analytical protocols: (a) the determination of isoflavones in plant tissue extracts, and (b) the analysis of bacterial growth curves. Since the protocol for isoflavone determination involves a multistep procedure that was standardized for all experiments, these methods will be described first, in sub-Sections 1 to 4; other experimental designs will follow, in Sections 5 & 6. Finally, Section 7 will describe the materials and methods for bacterial growth curves.

D.1. PLANT MATERIAL

D.1.1. Seed Cultivar

Seeds of white lupin, *Lupinus albus L. cv.* Kievskij were generously supplied by Dr Satoshi Tahara, Dept. of Applied Biosciences, Hokkaido University, Sapporo, Japan. All experiments using *L. albus* seedlings were carried out under controlled, sterile conditions. Except for nodule harboring-plants which were grown in the greenhouse, white lupin seeds were inoculated under normal conditions with a commercial powdered inoculum of *Rhizobium lupini*.

D.1.2. Seed Sterilization and Imbibition

Surface sterilization of seeds was carried out by standard procedure, which include the use of alternate soaking in organic solvent and alkaline solutions (Mulligan and Long, 1985; Hungria *et al.*, 1991b). However, the latter procedure proved to be insufficient with lupin seeds, which harbor a relatively big funiculus (the ridged hole on the seed coat), thus allowing endogeneous bacteria and fungi to survive such sterilization procedure.

Therefore, a more thorough sterilization procedure was used which consisted of flaming the seeds, with absolute ethanol for 10 seconds, followed by dropping in sterile water. About 100 seeds were then transferred to a 500 ml Erlenmeyer flask containing 250

mL of a water-bleach solution (4:1, v/v), containing 0.05% Tween 20 as a surfactant. They were rotated on a rotary shaker for 30 min at 250 rpm. The alkaline solution was then discarded and the 'bleaching' procedure was repeated once more. The bleached-off seeds were rinsed with four aliquots, 15 min each, of sterile water. Finally 250 mL of setrile water was added to the seeds and the flask was rotated (175 rpm) for 24 hr at room temperature, in order to allow seed imbibition and aeration. All steps following ethanol treatment were done under aseptic conditions. In all following experiments, the onset of germination (T=0) started with the imbibition and aeration of surface sterilized seeds.

D.1.3. Seed Germination

Following the 24 hr imbibition, seeds were allowed to germinate on sterile vermiculite, in an incubator at a light intensity of 350 to 400 $E \cdot m^{-2} \cdot s^{-1}$ (16/8 hr, light/dark, 26°/20°C) and 50% relative humidity. Note that 'incubation' will refer, thereafter, to the latter conditions. For this set up, the dry vermiculite (1.5 L), overlaying gravel (750 ml), was contained inside transparent polycarbonate boxes (28x17x12 cm). Distilled water (2.0 L) was added to swell the vermiculite before this set up was sterilized (121°C, 22 min, 20 psi). The 24 hr-soaked seeds were transferred aseptically and placed on the sterile vermiculite with the emerging root facing down.

This procedure enabled the production of sterile lupin seedlings with straight (10 to 25 mm long) emerging roots. This allowed easy handling of the seedlings for mounting on grids to collect exudates and/or to stand in Eppendorf tube for feeding radiolabeled precursors.

D.1.4. Dissection of Seedling Parts

Dissection of seedling parts or organs (Appendix A) was performed with a sterile scalpel on a flamed glass surface. The 'emerging root' includes the entire root system from the hypocotyl down to the root tip. The 'primary root tip' refers to the root at T=24 to 48

hr, or to the 5-7 mm growing tip at later growth stages, and includes the apical meristem of the root. The 'primary root distal' refers to the region adjacent to the hypocotyl (T=96 hr), but without root hairs. It is the region from which lateral roots and nodules will emerge later. The 'primary root proximal' is the region in between the primary root distal and primary root tip (T=72 hr). This region harbors root hairs, where the lateral roots did not yet emerge but where *Rhizobium* infection takes place. The 'lateral root tip' is the whole lateral roots (T=day 7 to 10 days), or the 5 mm long apical meristem of lateral roots at later growth stages. The 'lateral root proximal' is the region between the primary root distal and the lateral root tip, where the nodules will appear. The 'hypocotyl' refers to the region below the cotyledons and above the root system, whereas the 'epicotyl' constitutes all of the aerial parts above the cotyledons. The 'cotyledons' represent the bulk of the seed, which is covered by the 'seed coat'. Finally, the 'exudate' constitutes what is secreted from the root system into the medium, but excludes the 'effusate' derived from the seed coat and cotyledons during imbibition.

D.2. NUTRIENT SOLUTIONS AND STRESS MEDIA

D.2.1. Johnson's Medium

The original Johnson's nutrient solution (Johnson *et al.*, 1957) was modified to contain similar molarities of all elements, and all nitrogen-containing salts were replaced by nitrogen-free salts. The new formulation consisted of (a) Macronutrients: KH_2PO_4 (1.0 mM), KCl (2.0 mM), $CaCl_2$ (5.5 mM), $MgSO_4 \cdot 7H_2O$ (0.5 mM), Fe-EDTA (50 mg/L (w/v)), (b) Micronutrients: H_3BO_3 (12.5 μM), $MnSO_4 \cdot H_2O$ (1.0 μM), $ZnSO_4 \cdot 7H_2O$ (1.0 μM), H_2MoO_4 (2.0 μM), $CuSO_4 \cdot 5H_2O$ (0.26 μM). The pH of the medium was adjusted to 6.5 with 1.0 M KOH solution. The medium was filter-sterilized under vacuum, and will be referred to as the 'Control' medium throughout the text.

D.2.2. *Rhizobium lupini* Suspension Medium

Elicitation with *Rhizobium lupini* was accomplished by diluting a 20 ml culture of this strain with Control medium 1/50 (v/v) (Section D.2.1.). This solution was also used as a nutrient growth medium for *L. albus* seedlings (see D.6.3.), and is referred to as '*R. lupini* Suspension' medium throughout the text.

In order to obtain the bacterial inoculum, a single colony of *R. lupini* was grown at 28°C on a rotary shaker to an OD_{600} of 1.2 in liquid YEM medium. The latter consists of 0.4 g/L yeast extract, 10.0 g/L mannitol, 0.05% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.01% NaCl (w/v)(Vincent, 1970). The latter mixture was diluted with Control medium 1/50 (v/v), so that the optical density resulting from bacteria in this medium reached an OD_{600} of 0.024.

D.2.3. Yeast Extract Medium

Yeast extract was sterilized in water at a concentration of 10% (w/v), then diluted with sterile Control medium 1/10 (v/v) and used as a stress incubation medium. It will be designated later in the text as the 'Yeast Extract' medium.

D.2.4. Chitosan Medium

Chitosan (ß-1,4 linked glucosamine polymer, Sigma) was solubilized in 0.1% aqueous acetic acid (HOAc), and the pH of this solution was adjusted to pH 6.0 with a solution of concentrated sodium acetate (NaOAc) without the precipitation of polyglucosamine. Buffered solutions containing chitosan at 0.05%, 0.005% and 0.0005% (w/v) were used as stress media. A NaOAc-buffered 0.1% aqueous acetic acid solution (final pH=6) was also used as a control incubation medium. This control solution will be referred to in the text as the 'Chitosan control' medium.

D.2.5. Copper Chloride Medium

$CuCl_2$ was solubilized in water at a concentration of 0.3M, and filter sterilized. Dilutions of this stock solution in sterile water were made aseptically to yield the desired concentrations. $CuCl_2$ was used as abiotic elicitor at concentrations of 3.0, 0.3 and 0.03 mM. Since the pH of these solutions closely averaged 4.3, a control solution was made up of distilled water adjusted to pH 4.3 and used as the '$CuCl_2$ control' medium.

D.3. FLAVONOID EXTRACTION AND SAMPLE PREPARATION

D.3.1. Root Systems, Seedling Parts and Nodules

Whole root systems, seedling parts, and nodules were harvested at the appropriate time intervals (Sections D.5 and D.6), dissected and weighed on an analytical balance (detection limit=100µg). They were homogenized in Eppendorf tubes with washed sand, and an appropriate volume of 80% MeOH using a glass rod The resulting homogenate was spun in an Eppendorf microfuge for 2 min at 10,000 rpm. The supernatant was collected, the pellet re-extracted with 80% MeOH, and finally with absolute MeOH. This extraction procedure allowed the solubilization of glucosides, aglycones and prenylated derivatives. The combined methanolic extracts were evaporated in a Speed Vac concentrator, and the residue was resuspended in an aliquot of 80% MeOH proportional to the weight of the extracted tissue. The final extract was centrifuged for 2 min prior to HPLC injection. As a standard procedure, 20 µL of this extract was used for HPLC determinations. This was made equivalent to 50 mg f.wt. of seed coat or cotyledon tissue, or to 5 mg f.wt. of other tissues or nodules.

D.3.2. Exudates

Root exudates were collected at appropriate time intervals (Sections D.5. and D.6.). They were adjusted to pH 4.5 to 5.0 with 1N HCl and subjected to liquid-liquid extraction,

twice with an equal volume of ethyl acetate. The combined organic layers were evaporated *in vacuo* at 40°C. The dry residue was dissolved with 80% and 100% MeOH, and the combined methanolic extracts were lyophilized in a Speed Vac concentrator. The resulting residue was redissolved in a measured volume of 80% MeOH, where 20 µL extract was equivalent to the exudate of 3 seedlings. As a standard procedure, 20 µL of this methanolic extract was used for HPLC analysis.

D.4. ANALYSIS OF ISOFLAVONOIDS BY HPLC

D.4.1. HPLC Equipment

The HPLC system used (Waters™, Millipore™) consisted of a dual M510 solvent delivery system, a M490E programable multi-wavelength absorbance detector, a 7010 Rheodyne sample injector (1000 µL loop), and a System Interface Module (SIM) for data aquisition. Data aquisition was carried out using the Baseline 810 software operated on an IBM compatible, 25 mHz 386SX 4Mb/80Mb personal computer, that was upgraded with a math co-processor and two analog input boards.

D.4.2. Elution Protocol

Isoflavonoids were chromatographed on a Merck™ reverse phase C18 LiChrospher 100 column, 250 x 4 mm I.D. (particle size, 5 µm). Chromatography was carried out at a flow rate of 1mL/min. Initial conditions of elution consisted of 45% solvent A (0.5% methanolic acetic acid), in 55% solvent B (0.5% aqueous acetic acid) for 2 min. This was followed by a gradient increase to 100% solvent A in 23 min, then isocratic conditions were maintained for a further 10 min. Finally, initial conditions were attained in 5 min and the column was allowed to equilibrate for an additional 15 min before a new sample was injected.

D.4.3. HPLC Analysis

Both qualitative and quantitative determinations, as well as the calibration curves of different isoflavonoids, were achieved using the capabilities of the chromatography software Baseline™ 810. Qualitative analysis (peak identification) is based on the retention time (Rt), previously determined with reference compounds, and the proportional absorbance of peaks at different wavelenghts, i.e. simultaneous absorbances at 254nm, 280 nm, 340nm and the ratio of 280/340nm (Table 2). These two parameters enabled consistent identification of isoflavonoid peaks. Quantification was achieved on the basis of the surface area (μV/sec) of individual peaks at 254 nm. Empirical extinction coefficients were determined (Table 2) on the basis of several replicate injections of known amounts of authentic compounds. Calibration curves were thus generated and linearity was observed within a concentration range of 10 ng to 5 μg.

D.4.4. Data Processing

The data from each analyzed sample was retrieved from the Baseline software, in the format of a 'peak integration report'. These reports included surface areas of eluted peaks (μV/sec at 254 nm) and their retention times. Surface area values of interest (12 peaks were processed in these analyses, see Table 2) were entered manually to generate a 'data base' which included all the data for each experiment (Sections D.5. and D.6.).

Data processing was achieved using the capabilities of the software EXCEL™ 2.1 on a Windows™ 2.1 / DOS™ 3.0 support system. EXCEL is a software program which can mathematically process many calculations simultaneously. It comprises spread sheets composed of lines (labeled with numbers) and columns (labeled with letters) which can be referred to as appropriate address formulations. Mathematical formulae are typed in a background fashion together with the appropriate addresses, and the resulting values (originating from calculations on designated addresses) appear in the foreground.

Table 2: Physicochemical characteristics of lupin isoflavonoids

Compound[1]	Extinction coefficient[2] (nmol·sec^{-1}·v^{-1})	Maximum absorbance[3] (nm)	Relative absorbance[4] (280/340nm)	Molecular weight (g/mol)	Retention time (min)
Genistein	4.03	260.6	3.00	270.23	14.7
2'-Hydroxygenistein	6.22	259.0	3.05	286.23	11.7
Lupinalbin A	2.03	256.0	0.78	285.23	19.7
Genistein-7-*O*-glucoside	0.67	261.4	3.68	432.39	6.2
2'-Hydroxygenistein-7-*O*-glucoside	0.89	259.4	3.90	448.39	3.7
Wighteone	1.49	267.0	5.79	338.23	22.3
Isowighteone	1.51	262.3	4.94	338.23	22.7
Lupiwighteone	1.00	265.8	3.25	338.23	23.1
Licoisoflavone A	0.67	261.6	4.43	354.23	20.7
Luteone	1.01	264.4	4.68	354.23	20.4
Lupalbigenin	0.98	267.6	6.56	406.23	26.1
2'-Hydroxylupalbigenin	1.06	267.8	6.13	422.23	25.4

[1]Structural formulae are shown in Figure 2.

[2]Since no extinction coefficients are yet available for these compounds, relative extinction coefficients were determined from several replicate HPLC injections at different concentrations (Section D.4.3.). Units are shown in nanomoles per second per volt, at 254 nm.

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

[4]Relative absorbance was deduced from spectral data of standard compounds.

Spread sheets (data bases) were designed such that the data derived from one chromatogram occupies a single line, whereas different columns refer to parameters of extraction, such as run number, growth conditions, growth stage, sample size, sample weight, quantity injected (columns A to L); or to the surface area of a given compound (columns M to AH). However, the first and second lines were kept for headings (of each column) and for individual extinction coefficients relevant to each compound. I shall further refer to a set of values under the designation of 'field'. For example, an experiment designed to compare a given treatment to that of the control includes triplicate injections and sampling at days 4, 7 and 10 with a total of 18 determinations, would therefore be processed as follows: Lines 1 and 2 are occupied by headings and extinction coefficients. Lines 3 to 20, columns A to L contain information relative to each extract (i.e. the 'raw data' field), while lines 3 to 20, columns M to AH contain the surface areas for individual compounds (i.e. the 'surface area' field), with a total of 180 peak values. Then, a 'concentration' field is generated (lines 23 to 40, columns M to AH) with mathematical calculations from three values: (a) the surface area field, (b) the quantity injected for each sample, i.e. a line and (c) the extinction coefficient of each compound, i.e. a column.

Calculations of isoflavone concentrations in lupin tissues were carried out as shown below. Note that in this sample calculation, (X) is the extinction coefficient of a compound (a column heading), (Y) is the quantity injected in a chromatogram (a line) and (X,Y) is the surface area of an individual peak.

$$\text{Concentration} = \frac{\text{extinction coefficient (X) x surface area (X,Y)}}{\text{quantity injected (Y)}}$$

$$\text{Concentration} = \frac{[(\text{nmol x sec})/\mu\text{V}) \text{ x } (\mu\text{V/sec})]}{(\text{mg f.wt.})} = \text{nmol/mg f.wt.}$$

$$\text{Concentration} = \text{nmol/mg f.wt.} = \mu\text{mol/g f.wt.}$$

The above example applies to data treatment of tissue extracts. However, in the case of exudates, since no weight is avalaible, the concentration values are calculated on the basis of the amount exuded per seedling (eps). Therefore:

$$\text{Concentration} = \frac{\text{extinction coefficient (X) x surface area (X,Y)}}{\text{quantity injected (Y') x number of seedlings (Y")}}$$

$$\text{Concentration} = \frac{[(\text{nmol x sec})/\mu\text{V})\text{x } (\mu\text{V/sec})]}{\text{(a fraction) x (number of seedlings)}} = \text{nmol/eps}$$

Concentration = nmol/eps = nmol exuded per seedling (for exudate)

Further processing of data is achieved with the formation of a new field, the 'group concentration' field (lines 23 to 40, columns AI to AW, in the case of the example consisting of 18 determinations). This field includes sums derived from values in the 'concentration' field for each substitution group described in Appendix B. For example, the concentrations of the glucosides of both genistein and 2'-hydroxygenistein (for chromatogram Y) are given in a column designated glucosides in the 'group concentration' field. Since this step involves a simple addition, no sample calculation is given.

An average field (line 41 to 49) is then derived from the concentrations of each replicate, for both the 'concentration' field (columns M to AH) and the 'group concentration' field (columns AI to AW). It should be noted that the number of lines is shrinking at this point; this is due to the fact that the 'average' field reduces the amount of values by a factor of 3 (in the above example).

Similarily, a 'standard deviation' field is calculated from the concentration field (lines 50 to 59) for both groups (columns AI to AW) and individual compounds (columns M to AH). Both formulae for the mathemetical average and standard deviation are shown on top of the next page:

$$\text{Mathemetical Average} = \underline{X} = \frac{(x1 + x2 + x3 + x_i + x_n)}{n}$$

$$\text{Standard Deviation} = sx = [(\Sigma x^2 - n\underline{X}^2)/(n-1)]^{-1/2}$$

Finally, both averages and standard deviation fields were transformed into relative abundance (%) as compared with the sum of all 12 isoflavones (the total isoflavonoids identified, a column in the 'group average' field). Thus, the former were used to generate the '% average' and the '% standard deviation' fields, respectively. Data bases will be identified by their respective names in Sections D.5 and D.6, and given an estimate of their sample size. Their units (nmol/mg f.wt., nmol/eps, or %) will be given in the Results (Section E).

D.5. INCORPORATION STUDIES WITH A RADIOLABELED PRECURSOR

D.5.1. Radiolabeled Chemicals: Source and Synthesis

[2-^{14}C] *Trans*-cinnamic acid was prepared from [2-^{14}C] malonic acid, by Dr J. Grandmaison, following the procedure of Vorsatz (1936). The synthesis was carried out with equimolar amounts of labeled [2-^{14}C] malonic acid (specific activity· 56mCi/mmol, New England Nuclear, Boston, MA) and benzaldehyde, dissolved in 0.3 ml pyridine and 10 µL aniline. The mixture was heated in an oil bath for 7 hr at 55°C. A 0.5 mmol of carrier malonic acid was then added and the heating continued for a further 7 hr. Dilution with water and acidification with HCl yielded cinnamic acid, which was dissolved in MeOH, and purified by preparative thin layer chromatography. The final purity of the product was assessed by a UV spectrum and co-chromatography with a commercial sample of cinnamic acid and autoradiography. The radioactivity per unit volume was estimated to approximately be 14,000 dpm/µL.

D.5.2. Experimental Design

A total of five isotope feeding experiments, designated A to E, were performed and the protocols for their treatment are outlined in Appendix D. A summary for each experiment is given in Section D.5.3. Treatments A and B were pulse-labeling studies whereas the others (treatments C, D and E) were pulse-chase labeling studies

Sterile, 2-day-old seedlings (Sections D.1.1., D.1.2. and D.1.3.) were used for all incorporation studies. Each feeding experiment was conducted under aseptic conditions, and the amount of label, the incubation period, the number of seeds used for each treatment, as well as the number of determination are shown in Appendix D. For root feeding (treatments A, C, D and E), the seedlings were placed up-right in an Eppendorf. For segment feeding (treatment B), the seed coat was gently taken away, while both emerging root and cotyledon were separated, cut into pieces aseptically and placed in Eppendorf tubes. In all experiments, the radiolabeled precursor was administered through a cotton-wool wick, placed in the bottom of the Eppendorf. After the label was taken up, small volumes of sterile water were added to the seedlings in order to prevent their desiccation, until the end of the incorporation period. The tissues were removed, thoroughly rinsed with distilled water and extracted, and the determination of isoflavonoids done according to Sections D.3. and D.4. After extraction, the radioactivity of the methanolic extracts were scintillation counted, in order to determine the percentage label incorporation in the soluble fractions relative to the amount of label fed (Appendix D).

Twenty drop-mode fractions from HPLC eluates were collected in scintillation vials, correlated to precise retention times, and allowed to evaporate in a fume hood before scintillation counting. Disintegrations per minute (dpm) and surface areas (μV/s) generated from each determination, were entered manually in EXCEL™. A special data base was prepared to correlate fractions with peaks in the elution profile, and calculate the relative abundance (%) of both radioactivity and concentrations for groups of (or individual)

compounds. The results were expressed as nmol/mg f.wt. or nmol/eps and percentage dpms to total dpms in fractions corresponding to the identified peaks.

D.3.3. Summary of Feeding Experiments

A- An intact seedling was administered 1μCi (160 μL) of [2-^{14}C] cinnamic acid for 48 hr.

B- Each excised seedling part was administered 0.31 μCi (50 μL) of [2-^{14}C] cinnamic acid for 24 hr.

C, D, E- Four intact seedlings were administered 0.25 μCi (40 μL) of [2-^{14}C] cinnamic acid, for 24 hr (i.e. T= 48 to 72 hr). At the end of the incorporation period, they were segregated into two sets, each set was immersed in test tubes containing: (D) water, (E) a solution of 0.03 mM $CuCl_2$ (Section D.2.5) and (F) a *Rhizobium lupini* suspension (D.2.3; final OD_{600}=0.14); and incubated for 7 more days under aseptic conditions.

D.6. DETERMINATION OF ISOFLAVONOID CONTENT: EXPERIMENTAL DESIGN

D.6.1. Distribution in Different Parts of the Seedling

Seedlings, 48 hr-old, were incubated in vermiculite to which the Control medium was added whenever required. Samples were harvested at the designated periods shown in Appendix A. A pool of three seedling parts was used to generate each sample extract (Section D.3.1.), and chromatography was carried out as described in Section D.4. The data base generated from this experiment is designated A6.XLS, and includes 76 determinations, comprising 912 peak values.

D.6.2. Composition of Exudates

Two sets of 38 seedlings were used for the recovery of their flavonoid exudate, which were prepared as described in Section D.1.2. Soaked seedlings (T=24 hr) were mounted

aseptically on a stainless steel grid (5 x 5 mm mesh) over 10 cm glass Petri dishes filled with distilled water (100ml) inside a polycarbonate box. The whole set-up was incubated as described in Section D.1.2. and exudates were collected daily between 2 and 16 days, except day 11, and duplicate samples secured each harvest day (Appendix A). In addition, seed effusates (12- and 24hr-soaking water from the onset of germination) were also analyzed. Exudates and effusates were extracted and analysed by HPLC as described in Sections D.3.2. and D.4., respectively. The database generated from this experiment is designated A6.XLS and includes 37 determinations, comprising 444 peak values.

D.6.3. Isoflavonoid Accumulation and Secretion by Stressed Roots

Two days-old seedlings (Sections D.1.2. and D.1.3.) were mounted asceptically on stainless steel grids (5 x 5 mm mesh), inside polycarbonate boxes, over 5 cm glass Petri dishes containing 35 ml of the appropriate media under sterile conditions (Section D.2.). The whole set up was further incubated as described in Section D.1.2. The seedlings and media containing exudates were harvested at 4, 7 and 10 days; i.e., before, during and after lateral roots have appeared (Appendix A). Duplicate sets, each made up of three root systems and culture media of nine seedlings (per harvest time and per medium used), were extracted as described in Sections D.3.1. and D.3.2. respectively, and chromatography carried out as described in Section D.4. The database generated from this experiment is designated TRIGA.XLS for whole roots and TRIGB.XLS for exudates. These include 108 determinations, comprising 1296 peak values.

D.6.4. Isoflavonoid Content of Nodules

Four mature lupin plants (6 weeks old), grown in the greenhouse, were thoroughly washed with tap water, and their nodules were harvested and segregated on the basis of size (0-2, 2-4 and > 4 mm in diameter). Each size-group was randomly sub-divided into three samples. The nine resulting pools were weighed, extracted as described in Section

D.3.1., and samples of these extracts were injected into the HPLC as described in Section D.4. Duplicate samples of lateral roots of these plants, harboring nodules, were also sampled and analyzed. In addition, triplicate samples of 2 week old lateral root proximal were also analyzed (Section D.6.1). A database, designated NODULE3.XLS, was created from these determinations; it includes 14 determinations, comprising 168 peak values.

D.7. BACTERIAL GROWTH RATE IN THE PRESENCE OF FLAVONOIDS

D.7.1. Bacterial Strains

The growth rates of the following bacterial strains were determined in a complex medium containing various flavonoids at different concentrations.

Bacterial Strain	Source
Escherichia coli B	Ward's, #85W 0401.
Bacillus subtilis	Ward's, #85W 0228.
Rhizobium meliloti	ATCC 9930.
Rhizobium lupini *	ATCC 35173.
Agrobacterium rhizogenes **	LBA 9402.

D.7.2. Growth Curves of Bacteria

Triplicate cultures originating from different single colonies were grown overnight in liquid LB medium (4.1 g/L sodium chloride, 8.0 g/L tryptone and 4.0 g/L yeast extract). The *Rhizobia* were subcultured 1/20 and the other species 1/50 in LB medium. Aliquots (3 mL) were dispensed into sterile test tubes (150 x 25 mm I.D.) containing 30 µL of the flavonoid solution. The flavonoid compounds used were solubilized in 80% MeOH.

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* Listed as *R. loti* in the ATCC catalog, but since it was isolated from *Lupinus* species, it is designated as *Rhizobium lupini* throughout the text.

** Gift from by Dr M. P. Robins, Aberystwyth, UK.

Quercetin was dissolved in 50% dimethyl sulfoxide (DMSO). Two control curves were therefore established, one with 80% MeOH and the other with 50% DMSO. Each flavonoid compound was assayed at concentrations of 100 nM, 1.0 µM and 10.0 µM.

The different growth assay mixtures were incubated at 21°C (28°C for *Rhizobium* species) on a rotary shaker at 300 rpm. A lag period of 2 hr (12 hr for *Rhizobium* species) was allowed to lapse before sampling. Growth was monitored by measuring photometric absorbance at 600nm of a 1/10 dilution of the culture in disposable 1.5 mL cuvettes, at 90 min intervals.

D.7.3. Data Processing

A data base was developed for each strain and processed on EXCEL™. The values from each reading were entered manually, representing 3 replicates of 7 data points for each of the 5 strains tested in 3 concentrations of 14 flavonoid compounds (including control curves), thus generating 4410 readings. The growth rate was deduced from the logarithmic portion (i.e. the straight line portion of the growth curves on a log plot) of each triplicate curve, by calculating the regression slope of these data points. The slope averages of triplicate values were divided by that of the corresponding control, thus generating a 'relative growth rate'. If the latter differs from the control (=1.0), it means that a given compound has an effect on the bacterial growth rate (Appendix E).

To find out if variations among the relative growth rates were statistically significant, the regression slopes were compared using an ANOVA test (analysis of variance) with a confidence level of P (>0.95). In addition, a Student-Newman-Keuls (S.N.K.) test was performed to establish a scale of variations (i.e. which treatment is considered the most inhibitory). All statistical analyses were carried out using the SPSS™ software on a VAX computer (Norusis and SPSS, 1990).

E. RESULTS

E.1. PROTOCOL FOR HPLC DETERMINATIONS

An elution protocol was developed with the aim to acheive an unambiguous fractionation of isoflavone glucosides and aglycones as well as their prenylated derivatives. In addition, the use of a gradient-mode elution enabled the separation of aglycones and glucosides, as well as their mono- and diprenylated derivatives of both genistein and 2'-hydroxygenistein (Figure 6). This protocol enabled a reliable, qualitative and quantitative estimate of lupin isoflavonoids within a single chromatographic run.

The presence of two unknown isoflavone peaks in lupin tissue should be noted, since one of them was as abundant as the glucosides. These compounds, which were eluted after the glucosides and before the aglycones upon HPLC fractionation of root extracts, were not reported in the result section. It is indeed tempting to speculate on the molecular structure of these compounds since their ratio of absorbance (280/340 nm) suggests that they are isoflavones and their chromatographic behavior (Rt=8-10 min) indicative of being glucosides, although less polar than genistein 7-*O*-glucoside. They may, in fact, be both prenylated and glucosylated isoflavonoids, however further investigation of these peaks is required for their proper identification. Such prenylated glucosides have been reported to exist in white lupin (Shibuya *et al.*, 1991). Furthermore, other peaks were found which had a ratio of absorbance (280/340nm) similar to that of flavonoids (e.g. <1), as opposed to that of isoflavonoids (e.g. >1) and the chromatographic behavior of aglycones (Rt=11-17 min). These flavonoids occurred in relatively low quantities and were not indentified neither quantified in the present study.

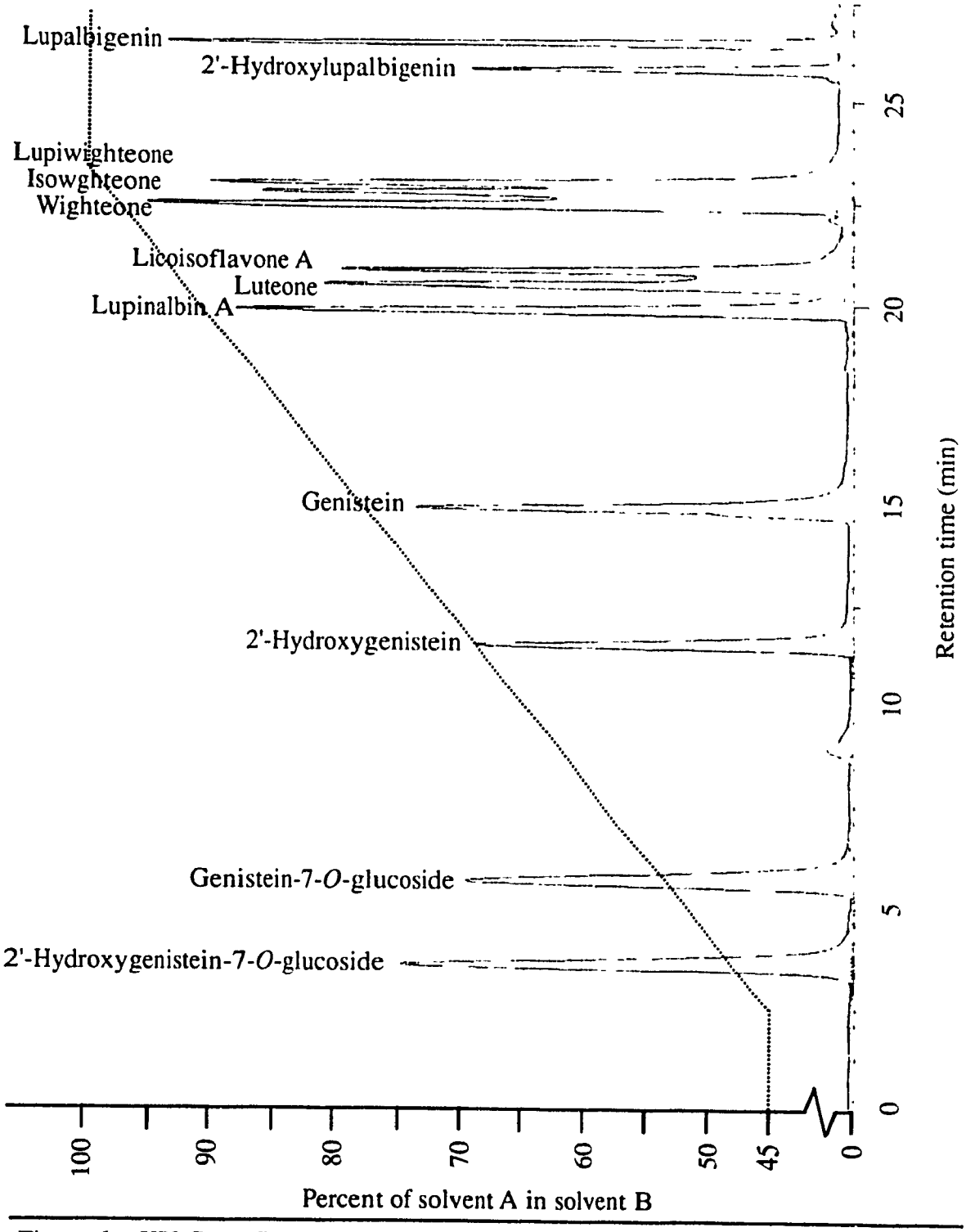

Figure 6: HPLC profile of reference compounds and elution protocol. Fractionation was achieved by increasing the percentage of solvent A (methanolic acetic acid 0.5% w/v) in solvent B (aqueous acetic acid 0.5% w/v) from 45% at Rt=2 min to 100% at Rt=23 min.

E.2. DISTRIBUTION OF ISOFLAVONOIDS IN DIFFERENT PARTS

E.2.1. Isoflavonoid Content of Seed and Seedling Parts

The isoflavonoid composition of the seedling parts was determined at different stages of early lupin development (Appendix A), and the HPLC profile of a lateral root tip extract is shown in Figure 7 as an example. These analyses aim at revealing the developmental variations within each part, and the differences in isoflavonoid pools among the different parts of the seedling. The most meaningful sampling stages will be presented in Table 3 (for dry seeds and soaked seed parts), Table 4 (for the primary root), Table 5 (for the lateral roots) and Table 6 (for the aerial parts). The detailed results of these analyses are also shown in Appendix C.

E.2.1.1. Analysis of Isoflavonoids in Seed Parts

The isoflavonoid content of dry seeds (e.g. including all seed parts except seed coats) and of the dissected parts following 24 hr-soaking (e.g. cotyledons, epicotyl, hypocotyl and primary root tip) are presented in Table 3. No data will be presented for the seed coat, since its isoflavonoid content amounts to a very low level (ca from 0.03 to 0.3 nmol/mg f. wt.). However, their relative abundance was almost similar to that of the cotyledons, except for the 2'-hydroxygenistein monoprenyls which amounts to 0.02 nmol/mg f. wt. in dry seed coats (i.e. 74.0 % of total isoflavonoids identified, Appendix C).

Dry seeds contained 0.2 nmol/mg f. wt. of each of the glucosides and the diprenyl derivatives which constituted the bulk of isoflavonoids, whereas the aglycones were less abundant (0 D, Table 3). The sum of monoprenylated compounds amounts to less than 0.002 nmol/mg f.wt. Given that lupin cotyledons are bulky in size, these results suggest the presence of a pre-existing pool of aglycones, glucosides and diprenyls, which accounts for their accumulation in this storage tissue.

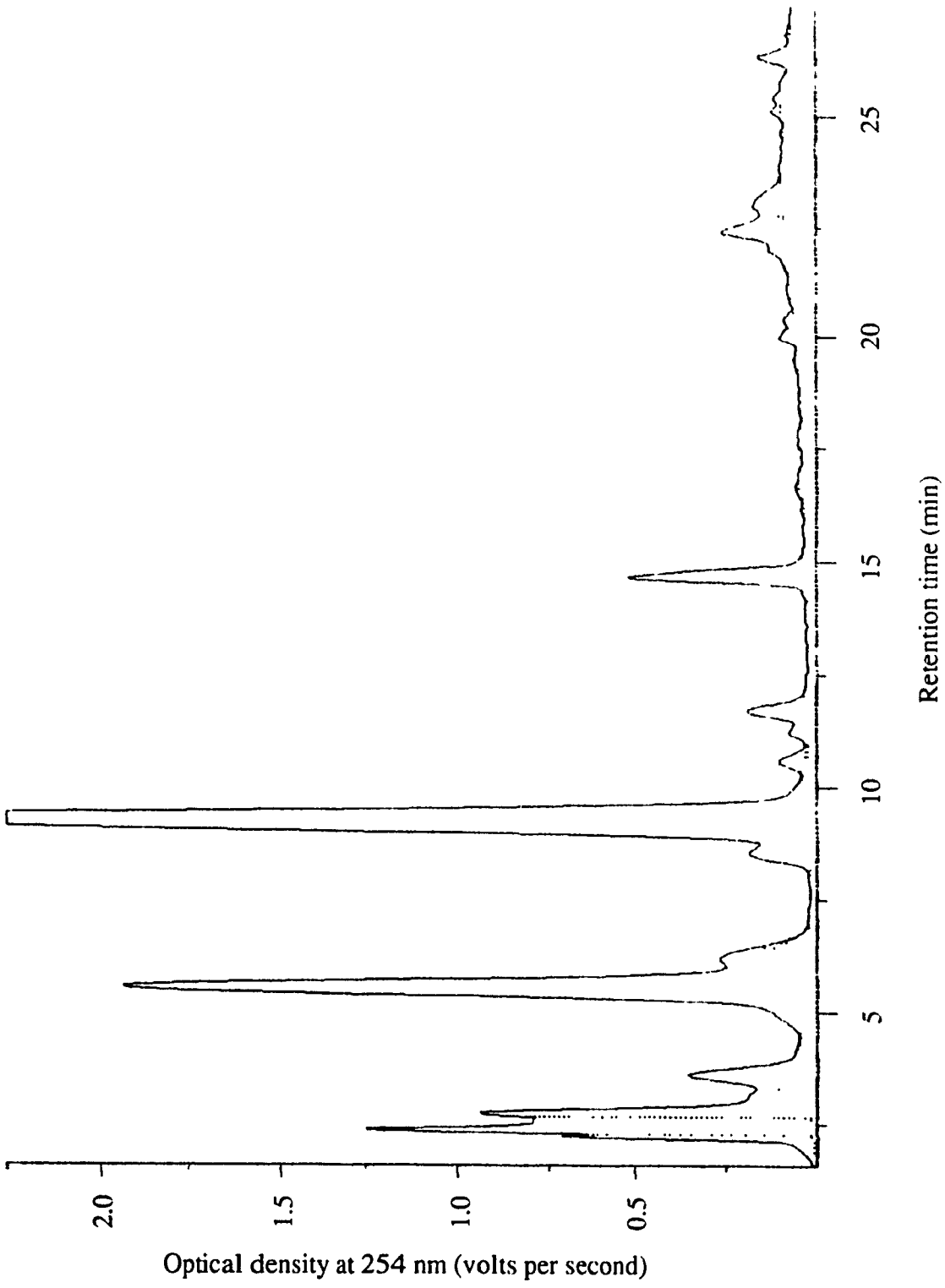

Figure 7: HPLC profile of seedlings lateral root tips at day 11.

Table 3: Isoflavonoid content of dry and soaked lupin seeds[1]

	Dry	Soaked			
	Seeds[2]	Coty -ledons	Primary root tips	Hypocotyls	Epicotyls
Isoflavonoid groups	0 D[3]	1 D	1 D	1 D	1 D
Glucosides	0.09	0.02[c]	0.46[f]	0.13[c]	0.22[a]
Aglycones	0.03	0.03[f]	0.12[f]	0.07[a]	0.09[a]
Gen. MonoPr.	0.00	0.00[a]	0.01[f]	0.04[f]	0.02[a]
2'-OH Gen. MonoPr.	0.00	0.00[f]	0.00[f]	0.00[c]	0.00[a]
Diprenyls	0.08	0.01[b]	0.00[f]	0.14[c]	0.24[a]
Total Identified[4]	0.20	0.06[e]	0.61[f]	0.39[b]	0.58[a]
Gen. Derivatives (%)	46.6	49.1[f]	67.8[f]	69.8[b]	71.6[a]

[1] Values are expressed in nmol/ mg f. wt. except for genistein derivatives* (percent relative abundance); they represent averages of duplicate samples, with standard deviations indicating confidence levels of: a, <5%; b, <10%; c, <15%; d, <20%; e, <25%; f, >25%.

[2] Dry seeds include cotyledons, hypocotyl, epicotyl and primary root tip.

[3] Abbreviations: D, days; Gen., genistein; MonoPr., monoprenyls (see Appendix B, for individual isoflavones included in each group).

[4] Totals include lupinalbin A (see Appendix B).

* The balance constitutes the 2'-hydroxygenistein derivatives (see appendix B)

Table 4: Isoflavonoid content of lupin seedling primary root[1]

	Primary root tip					
Isoflavonoid groups	-	2 D[2]	3 D	4 D	6 D	8 D
Glucosides	-	4.88^{e}	2.74^{a}	1.72^{f}	1.87^{e}	1.86^{e}
Aglycones	-	0.49^{f}	0.53^{f}	0.24^{f}	0.01^{d}	0.03^{f}
Gen. MonoPr.	-	0.06^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}
2'-OH Gen. MonoPr.	-	0.00^{f}	0.00^{e}	0.00^{f}	0.00^{f}	0.00^{f}
Diprenyls	-	0.37^{f}	0.01^{a}	0.01^{f}	0.01^{f}	0.03^{f}
Total Identified[3]	-	5.81^{e}	3.28^{b}	1.97^{f}	1.88^{e}	1.91^{d}
Gen. Derivatives (%)	-	97.0^{e}	96.1^{e}	91.3^{f}	86.7^{d}	85.0^{b}

	Primary root proximal					
Isoflavonoid groups	2 D	3 D	4 D	6 D	7 D	8 D
Glucosides	4.99^{f}	6.27^{e}	2.99^{f}	3.41^{e}	2.14^{f}	1.55^{f}
Aglycones	0.44^{d}	0.26^{a}	0.21^{f}	0.13^{f}	0.15^{f}	0.06^{f}
Gen. MonoPr.	0.02^{b}	0.00^{f}	0.03^{f}	0.01^{f}	0.03^{e}	0.02^{f}
2'-OH Gen. MonoPr.	0.00^{f}	0.00^{f}	0.00^{f}	0.01^{f}	0.07^{f}	0.06^{f}
Diprenyls	0.10^{e}	0.09^{f}	0.08^{f}	0.23^{f}	0.32^{f}	0.24^{f}
Total Identified	5.57^{f}	6.63^{e}	3.30^{f}	3.81^{e}	2.72^{f}	2.00^{f}
Gen. Derivatives (%)	98.4^{f}	98.7^{e}	91.8^{f}	89.7^{d}	79.9^{f}	77.9^{f}

.../continued

Table 4: Continued

	Primary root distal					
Isoflavonoid groups	2 D	3 D	4 D	6 D	7 D	8 D
Glucosides	ND	7.09[b]	5.09[f]	5.02[c]	4.51[c]	3.98[f]
Aglycones	ND	0.50[f]	0.25[f]	0.13[f]	0.14[d]	0.09[d]
Gen. MonoPr.	ND	0.00[a]	0.02[f]	0.03[f]	0.01[f]	0.01[f]
2'-OH Gen. MonoPr.	ND	0.00[f]	0.00[f]	0.01[f]	0.01[f]	0.01[f]
Diprenyls	ND	0.07[f]	0.04[f]	0.44[f]	0.17[c]	0.14[c]
Total Identified	ND	7.66[b]	5.41[f]	5.66[c]	4.84[c]	4.24[f]
Gen. Derivatives (%)	ND	97.5[b]	91.2[f]	86.4[c]	86.5[c]	87.9[f]

[1]Values are expressed in nmol/ mg f. wt. except for genistein derivatives (percent relative abundance); they represent averages of duplicate samples, with standard deviations indicating confidence levels of: a, <5%; b, <10%; c, <15%; d, <20%; e, <25%; f, >25%.

[2]Abbreviations: D, days; Gen., genistein; MonoPr., monoprenyls; ND, not determined (see Appendix B, for individual isoflavones included in each group).

[3]Totals include lupinalbin A (see Appendix B).

Table 5: Isoflavonoid content of lupin seedling lateral roots[1]

	Lateral root tip					
Isoflavonoid groups	7 D[2]	8 D	11 D	13 D	15 D	19 D
Glucosides	1.53[d]	1.58[d]	1.82[c]	2.02[b]	1.17[e]	1.21[c]
Aglycones	0.13[f]	0.12[f]	0.15[f]	0.08[e]	0.04[f]	0.19[f]
Gen. MonoPr.	0.00[f]	0.00[f]	0.12[f]	0.04[f]	0.07[f]	0.08[f]
2'-OH Gen. MonoPr.	0.01[f]	0.01[f]	0.03[f]	0.05[f]	0.07[f]	0.08[f]
Diprenyls	0.11[f]	0.09[f]	0.41[f]	0.13[f]	0.21[f]	1.79[f]
Total Identified[3]	1.77[d]	1.82[d]	2.53[f]	2.32[c]	1.56[f]	3.36[f]
Gen. Derivatives (%)	88.0[d]	79.3[b]	86.0[f]	70.8[c]	77.5[f]	84.6[e]
	Lateral root proximal					
Isoflavonoid groups	7 D	8 D	11 D	13 D	15 D	19 D
Glucosides	ND	ND	1.21[b]	0.90[b]	1.51[b]	0.77[d]
Aglycones	ND	ND	0.34[c]	0.08[f]	0.12[a]	0.24[f]
Gen. MonoPr.	ND	ND	0.02[a]	0.00[f]	0.00[d]	0.01[f]
2'-OH Gen. MonoPr.	ND	ND	0.00[e]	0.01[f]	0.03[e]	0.03[f]
Diprenyls	ND	ND	0.25[e]	0.07[b]	0.03[a]	0.46[f]
Total Identified	ND	ND	1.84[c]	1.08[c]	1.77[b]	1.54[f]
Gen. Derivatives (%)	ND	ND	84.1[c]	72.5[c]	77.3[b]	73.2[f]

[1-3], as in Table 4.

Table 6: Isoflavonoid content of lupin seedlings aerial parts[1]

		Cotyledons			
Isoflavonoid groups	-	2 D[2]	3 D	4 D	7 D
Glucosides	-	0.11[f]	1.95[f]	2.01[f]	1.06[e]
Aglycones	-	0.06[f]	0.68[c]	0.65[f]	0.24[f]
Gen. MonoPr.	-	0.00[f]	0.00[f]	0.01[f]	0.01[f]
2'-OH Gen. MonoPr.	-	0.00[f]	0.01[f]	0.01[f]	0.03[f]
Diprenyls	-	0.00[f]	0.00[f]	0.00[f]	0.00[c]
Total Identified[3]	-	0.18[f]	2.71[f]	2.74[f]	1.35[f]
Gen. Derivatives (%)	-	73.7[f]	48.7[d]	56.9[b]	75.4[d]

		Epicotyl			
Isoflavonoid groups	-	2 D	3 D	4 D	7 D
Glucosides	-	2.81[a]	10.34[b]	3.22[f]	3.42[f]
Aglycones	-	0.88[a]	0.72[b]	0.43[d]	0.49[b]
Gen. MonoPr.	-	0.00[a]	0.01[d]	0.02[b]	0.02[d]
2'-OH Gen. MonoPr.	-	0.01[a]	0.00[f]	0.00[f]	0.03[f]
Diprenyls	-	0.15[a]	0.25[b]	0.25[d]	0.01[f]
Total Identified	-	3.86[a]	11.34[b]	3.94[f]	4.00[f]
Gen. Derivatives (%)	-	91.4[a]	97.2[a]	92.6[f]	89.0[f]

.../continued

Table 6: Continued

	Hypocotyls				
Isoflavonoid groups	2 D	3 D	4 D	6 D	7 D
Glucosides	9.18[d]	11.5[a]	5.69[f]	4.02	4.11
Aglycones	0.55[c]	0.55[b]	0.42[f]	0.08	0.12
Gen. MonoPr.	0.01[e]	0.01[d]	0.02[f]	0.04	0.00
2'-OH Gen. MonoPr.	0.01[f]	0.01[f]	0.01[f]	0.01	0.01
Diprenyls	0.17[d]	0.17[a]	0.07[f]	0.01	0.01
Total Identified	9.92[d]	12.25[a]	6.20[f]	4.17	4.27
Gen. Derivatives (%)	94.4[d]	94.0[a]	92.3[f]	93.5	91.3

[1-3], as in Table 4.

Soaking of seeds resulted in a 3.4-fold decrease of total isoflavonoids in the cotyledons, which involved the glucosides and the diprenyls, whereas the amount of aglycones remained constant (1 D, Table 3). The total isoflavonoids present in the primary root tip, the hypocotyl and the epicotyl, were higher than those of the cotyledons (Table 3). These parts contained high proportions of each of the glucosides and the diprenyl derivatives, with lower levels of the aglycones (about 1/6 of total) (Table 3).

4.2.1.1. Analysis of Isoflavonoids in the Primary Root

The primary root was dissected into three parts, the tip and both the proximal and distal regions. The proximal region harbors root hairs and is suceptible to *Rhizobium* infection, whereas lateral roots and/or nodules will originate from the distal region. Lateral roots appear at day 7, at which time the epicotyl emerges from the cotyledons. Nodules only form before flowering of the host plant, that is 4 to 6 weeks old.

Primary root tip: The total isoflavonoids identifed in this part reached a maximum of 5.81 nmol/mg f. wt. at day 2, then decreased thereafter, with a significant decrease of diprenyls after day 2 (Table 4).

Primary root proximal: The total isoflavonoids identifed in this part reached a maximum of 6.63 nmol/mg f. wt. at day 3, and decreased gradually thereafter (Table 4). Both the aglycones and glucosides followed a similar trend, although they reached their maximum at days 2 and 3, respectively. Whereas the diprenyls were present in equal amounts from days 2 to 4, however, they increased by up to six-fold when lateral roots appeared (ca days 6 to 8). Of the monoprenylated compounds, low amounts of the 2'-hydroxygenistein group are only detected at days 6 to 8 (Table 4).

Primary root distal: Although the total isoflavonoids identifed in this part were higher than in the tip or the proximal region (7.66 nmol/mg f. wt. at day 3), they gradually decreased thereafter mainly at the expense of both glucosides and aglycones (Table 4). As compared with other primary root tissues, the distal region contained higher amounts (ca

<50%) of total isoflavonoids after day 4. Another difference from those other parts was the significant increase in diprenyls which reached a maximum of 0.44 nmol/ mg f. wt. at day 6, and decreased thereafter (Table 4).

However, it is interesting to note that the genistein derivatives constituted approximately 78 to 97% of the total isoflavonoids during germination and growth of the primary root. This seems to indicate a very low 2'-hydroxylase activity during early stages of development.

E.2.1.2. Analysis of Isoflavonoids in the Lateral Roots

The entire lateral roots were designated as lateral root tips at days 7 and 8, whereas later samples were dissected into two parts, the lateral root tip and its proximal region (Table 5).

Lateral root tip: The total isoflavonoids identifed in this part varied between 1.6 and 3.4 nmol/mg f. wt. (Table 5). As with most root tissues, it appears that the glucosides predominate over the other constituents and represent 72-87% of the total during development (days 7 to 15) and then decline thereafter. The aglycones and the monoprenyl derivatives represent less than 10% of the total at all the growth stages. However, there is a significant increase in the amount of diprenyls (4-fold) up to day 11, followed by another increase (15-fold) at day 19 (Table 5).

Lateral root proximal: A trend similar to that of the lateral root tip was observed for all isoflavonoid groups in this seedling part, except that the aglycones observed in the proximal region are higher (both in amount and in relative abundance) at day 11, as compared with lateral root tips.

The relatively high levels of genistein derivatives (70.0 to 88.0% of the total) found in both regions of lateral roots were similar to those of primary root tissues (Tables 4 and 5).

E.2.1.3. Analysis of Isoflavonoids in the Aerial Parts

Cotyledons: There was a dramatic increase (15-fold) in the amount of total isoflavonoids identifed in cotyledons, reaching a maximum of 2.7 nmol/mg f. wt. at days 3 and 4, which decreased thereafter at the expense of both the glucosides and aglycones. These maxima correlated with the appearance of chlorophyll in this tissue (days 3 to 4). Glucosides were the most abundant in this tissue, whereas aglycones constituted the remaining pool of isoflavonoids at all growth stages. This contrasts with the very low-detectable levels of prenylated compounds (Table 6). Of all the identified compounds, the genistein derivatives constituted 73.7% of the total at day 2, which decreased significantly to 48.7% with the appearance of chlorophyll, followed by a gradual increase during later stages of growth (Table 6). It is not known whether the cotyledons synthesized all the pool of compounds detected in this tissue or part of which was being translocated from the growing radicle, however, the data from pulse labeling studies tend to support the former (see Section D.5.).

Epicotyls: The total isoflavonoids identifed in epicotyls reached a maximum of 11.34 nmol/mg f. wt. at day 3, and decreased thereafter, mainly at the expenses of glucosides (Table 6). This maximum also correlated with the appearance of chlorophyll in this tissue. Both the glucosides and aglycones were most abundant in this tissue, except that the former were 2.5 to 7-fold higher than the latter. In addition, the amount of diprenyls increased up to day 4 and almost disappeared by day 7.

Hypocotyls: The total isoflavonoids identified in this part represent the highest level of all aerial parts reaching a maximum of 12.25 nmol/mg f. wt. at day 3, and correlated with the appearance of chlorphyll in this tissue, although they decreased gradually during growth (Table 6). However, their isoflavonoid composition was similar to that of the epicotyl, except that the decrease in diprenylated derivatives occurred at day 3 (Table 6).

It is remarkable to note that the genistein derivatives amounted to 89% to 94% of the total at all growth stages in both the hypocotyl and epicotyl, respectively, an extremely high ratio as compared with that of the cotyledons (Table 6).

E.2.2. Isoflavonoid Content of Exudates

The isoflavonoid composition of exudates was determined by HPLC (Figure 8) from 2 pools, each consisting of 38 seedlings (Appendix A) and sample collection was accomplished on a daily basis by replacing the exudate-containing water. Table 7 is a simplified version of the results of exudate analysis; the details of these results are shown in Appendix C. Table 7 presents the data for seed effusates during 24 hr soaking and root exudates over 12 days period; other sampling stages do not show any significant behavior.

Seed effusates: The total isoflavonoids identified increased from 2.8 to 4.4 nmol/mg f. wt. during the 12-hr period following the initial 12-hr soaking (Table 7). About one nmol/seedling of the glucosides and of diprenyl derivatives was detected in the effusates of the first 12 hr. At 24 hrs, the amount of aglycones has increased by 14-fold, both genistein- and 2'-hydroxygenistein monoprenyls increased by two-fold each; whereas the levels of glucosides and diprenyls decreased. The genistein derivatives, which constituted 55% of total isoflavonoids in 12-hr seed effusates, decreased to 33% during the following 12 hr (Table 7).

Root exudates: The total isoflavonoids identified in the exudates fluctuated in amount (ca 0.6-1.4 nmol/seedling) as germination proceeded until day 7, after which they significantly increased by 4- to 11-fold (Table 7). These increases involved all groups of isoflavonoids, especially the aglycones and their prenylated derivatives. Preceding these increases (e.g. days 2 to 7), however, the amounts of aglycones exuded daily decreased progressively starting with 0.5 nmol/seedling at day 2 to lower amounts thereafter, whereas the sum of prenylated compounds constituted about 50-60% of total isoflavonoids from day 2 to 7; the secreted amounts of both groups dramatically increased after day 7. Of the

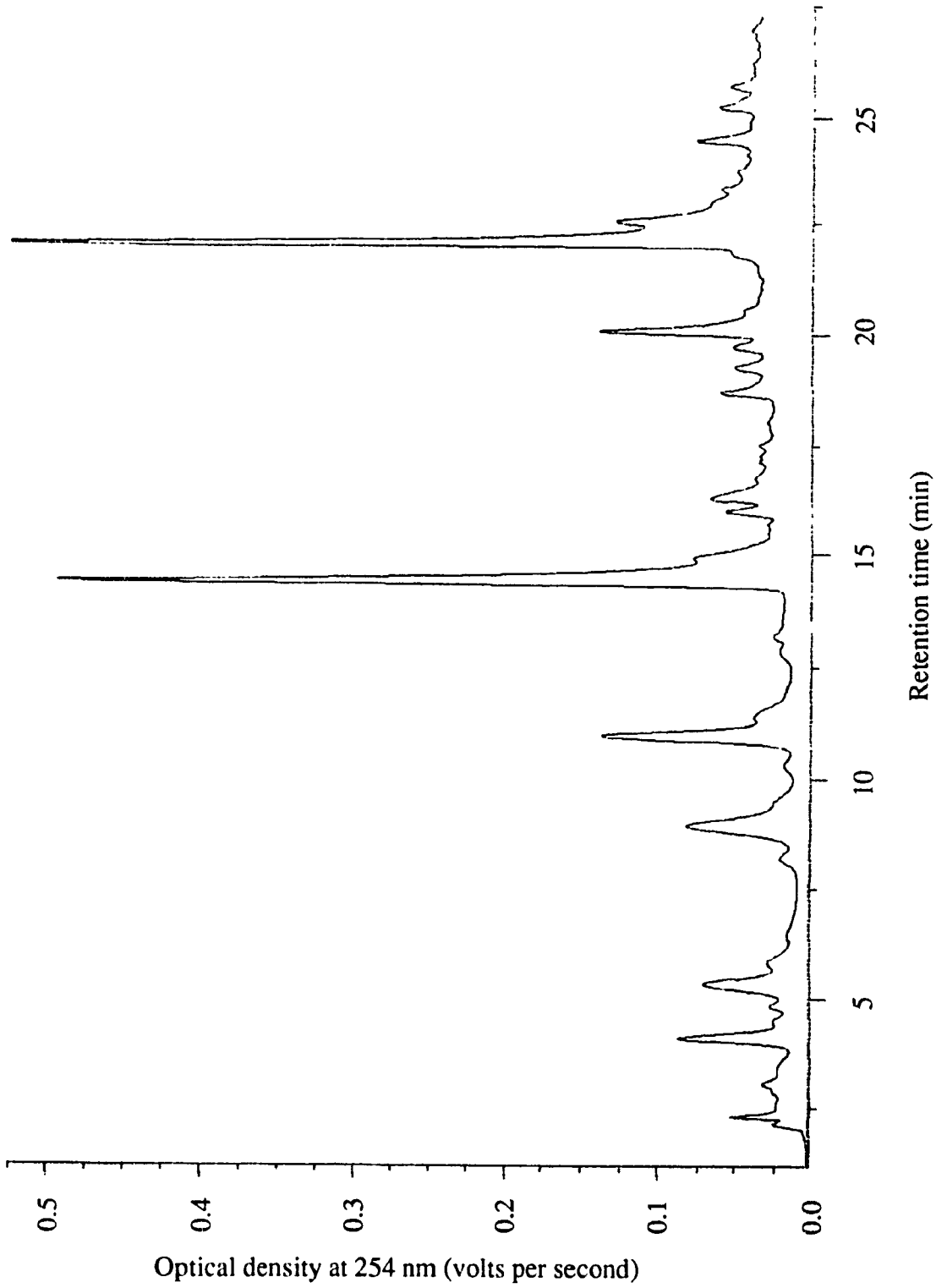

Figure 8: HPLC profile of exudates from days 3 to 4

Table 7: Isoflavonoid content of lupin seedling exudates[1]

	Seed effusate		Root exudate			
Isoflavonoid groups	0.5 D[2]	1 D	2 D	3 D	4 D	5 D
Glucosides	1.07	0.41^{f}	0.09^{f}	0.04^{b}	0.08^{f}	0.17^{f}
Aglycones	0.16	2.30^{c}	0.54^{f}	0.26^{f}	0.32^{f}	0.15^{f}
Gen. MonoPr.	0.45	0.66^{f}	0.14^{f}	0.19^{f}	0.86^{f}	0.40^{b}
2'-OH Gen. MonoPr.	0.23	0.58^{f}	0.07^{f}	0.03^{f}	0.08^{f}	0.06^{f}
Diprenyls	0.82	0.32^{f}	0.07^{f}	0.03^{f}	0.02^{f}	0.03^{e}
Total Identified[3]	2.77	4.40^{b}	0.96^{c}	0.56^{b}	1.38^{b}	0.81^{a}
Gen. Derivatives (%)	54.8	32.9^{f}	36.0^{f}	68.5^{f}	81.2^{f}	73.8^{e}

.../ continued

	Root exudate					
Isoflavonoid groups	6 D	7 D	8 D	9 D	10 D	12 D
Glucosides	0.18^{d}	0.20^{f}	0.33^{d}	0.25^{f}	0.58^{f}	0.72^{e}
Aglycones	0.15^{d}	0.18^{f}	0.75^{c}	1.41^{f}	2.04^{d}	1.98^{c}
Gen. MonoPr.	0.41^{b}	0.44^{f}	1.73^{f}	2.00^{c}	1.68^{f}	3.81^{d}
2'-OH Gen. MonoPr.	0.04^{d}	0.06^{f}	0.31^{f}	0.81^{f}	1.95^{f}	1.02^{d}
Diprenyls	0.05^{e}	0.08^{f}	0.94^{f}	0.52^{d}	0.60^{f}	2.00^{e}
Total Identified	0.88^{d}	0.98^{f}	4.23^{f}	5.32^{f}	7.43^{f}	11.22^{d}
Gen. Derivatives (%)	76.1^{c}	69.7^{f}	44.2^{f}	34.7^{d}	38.2^{f}	47.7^{d}

.../continued

Table 7: Continued

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[1]Values are expressed in nmol/seedling except for genistein derivatives (percent relative abundance); they represent averages of duplicate samples, with standard deviations indicating confidence levels of: a, <5%; b, <10%; c, <15%; d, <20%; e, <25%; f, >25%.

[2]Abbreviations: D, days; Gen., genistein; MonoPr., monoprenyls (see Appendix B, for individual isoflavones included in each group).

[3]Totals include lupinalbin A (see Appendix B).

prenylated compounds, the genistein monoprenyls secreted from lupin roots were the major constituents, especially during days 6 to 8 where they constituted almost 50% of the total. It maybe noted that the amount of glucosides exuded daily were relatively low as compared to the isoflavonoid content of seedling; they were secreted in gradually increasing amounts as seed germination proceeded (Table 6).

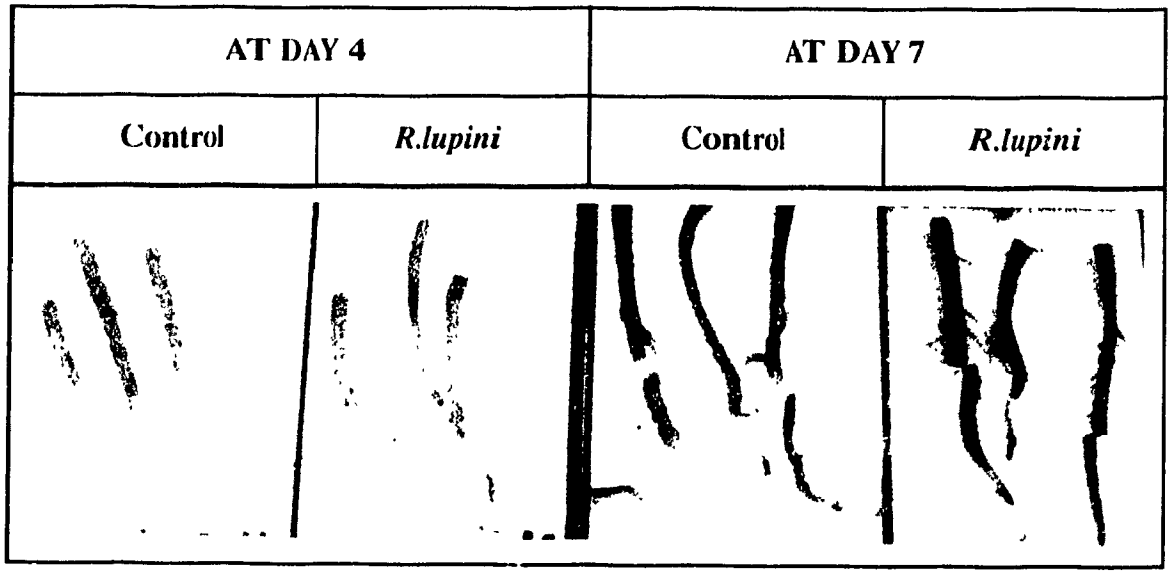
These results suggest a state of active secretion during seedling development and particularily the period corresponding to lateral root emergence (days 6 to 8). It is interesting to note that the ratio of genistein-derived isoflavonoids exhibited a significant increase during seedling development until day 6, then gradually decreased by day 12. This pattern is reflected in the dramatic increase in 2'-hydroxygenistein derivatives at the later sampling stages.

E.3. ACCUMULATION AND SECRETION OF ISOFLAVONOIDS UNDER STRESS CONDITIONS

The solubilization properties of two elicitors, chitosan and $CuCl_2$, required the adjustment of the buffering capacity of the incubation medium, or resulted in an altered pH upon solubization, respectively. Therefore, the results presented in this Section will be compared with the controls relevant to each elicitor used.

Photographs of stressed seedlings, shown in Figure 9, document the following morphological observations. As with the controls, seedlings treated with a *Rhizobium lupini* suspension or with yeast extract did develop lateral roots from days 7 to 10 (Figure 9-A, -B). However, seedling roots treated with yeast extract appeared swollen at day 4 and developed shorter lateral roots between days 7 and 10 as compared with the control; in addition, tissue browning took place (Figure 9-B). Chitosan-treated seedlings (Figure 9-C) had not developed lateral roots by day 10, and their root tissue was whitish in color as compared with control seedlings (e.g. controls in Figure 9-A and 9-B). These specific

9-A: Lupin seedling-roots elicited with *Rhizobium lupini*



9-B: Lupin seedling-roots elicited with yeast extract

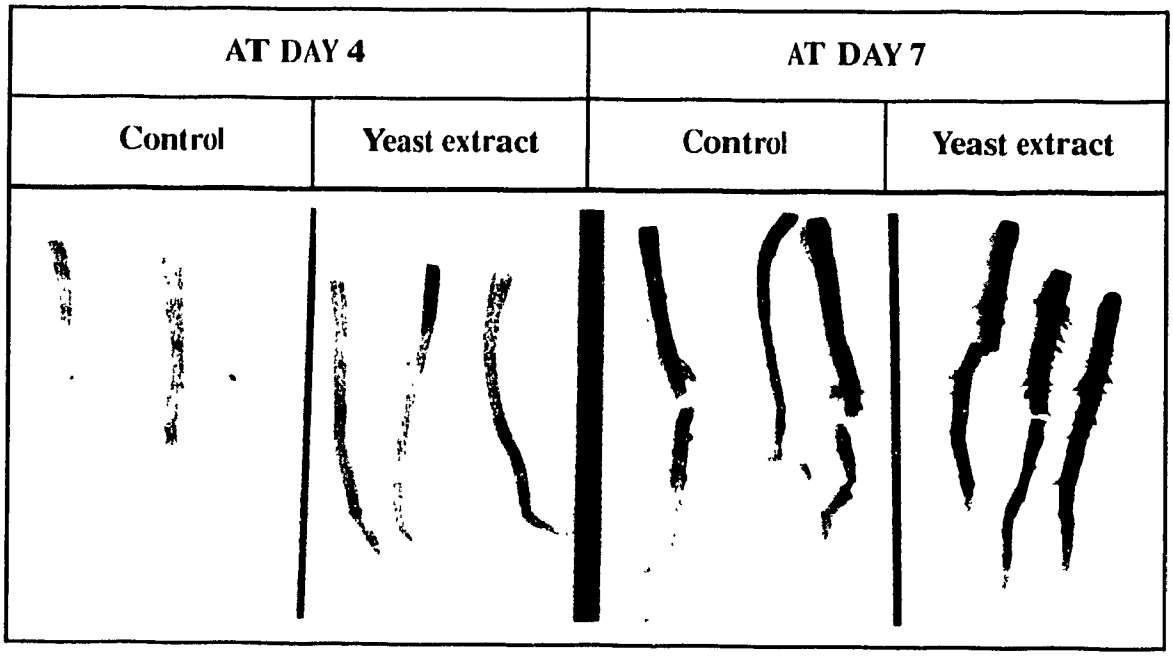


Figure 9: Photographs of lupin seedlings stressed with biotic and abiotic elicitors

9-C: Lupin seedlings elicited with chitosan

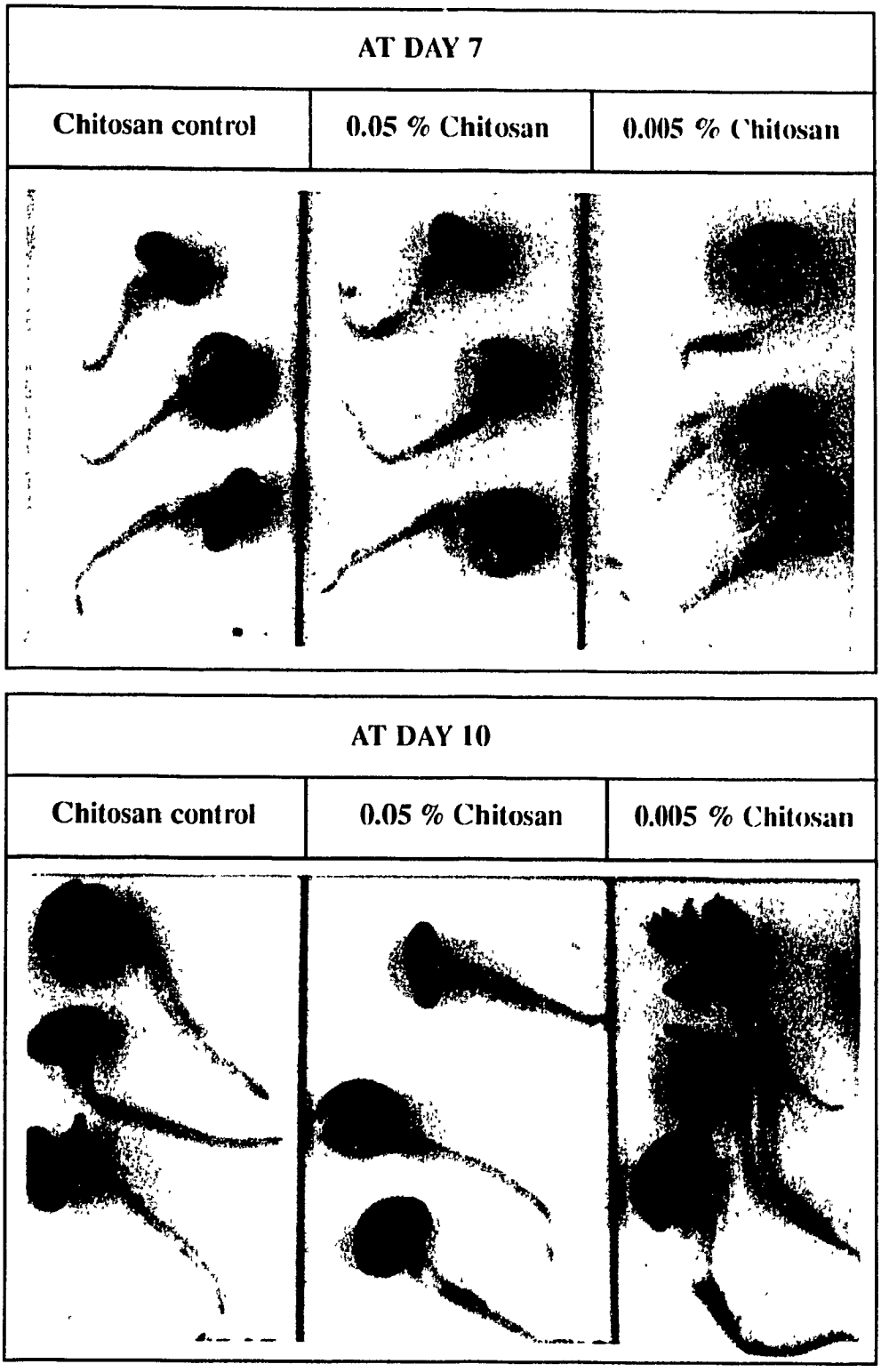
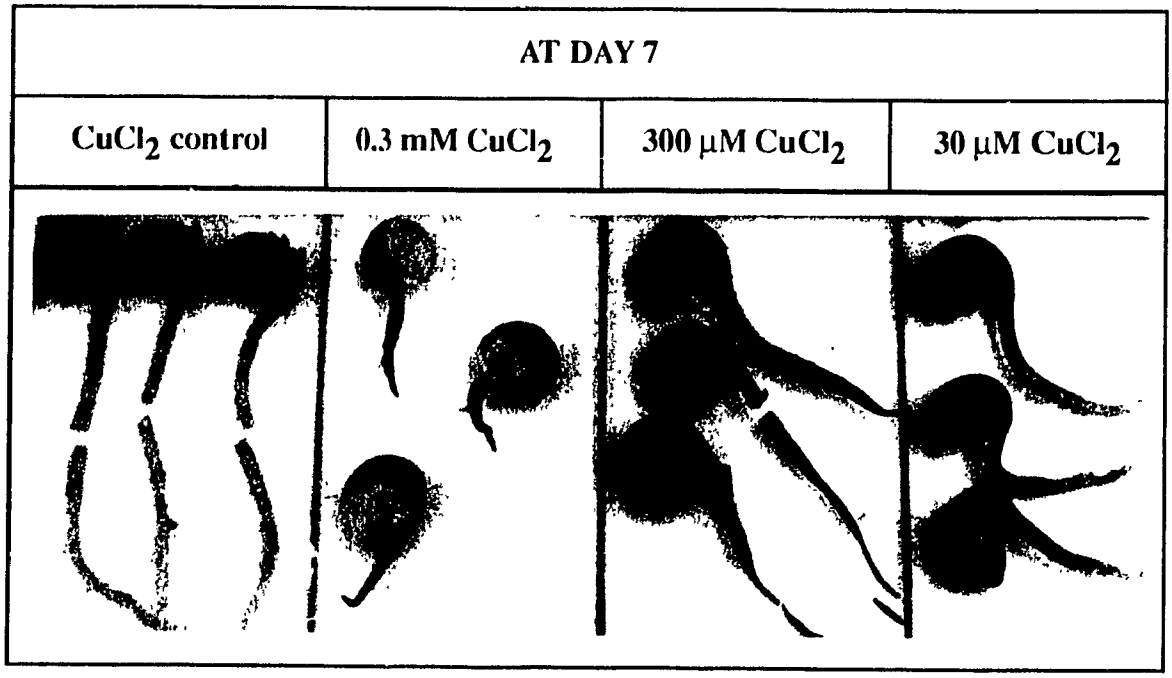


Figure 9: Continued

9-D: Lupin seedlings elicited with $CuCl_2$



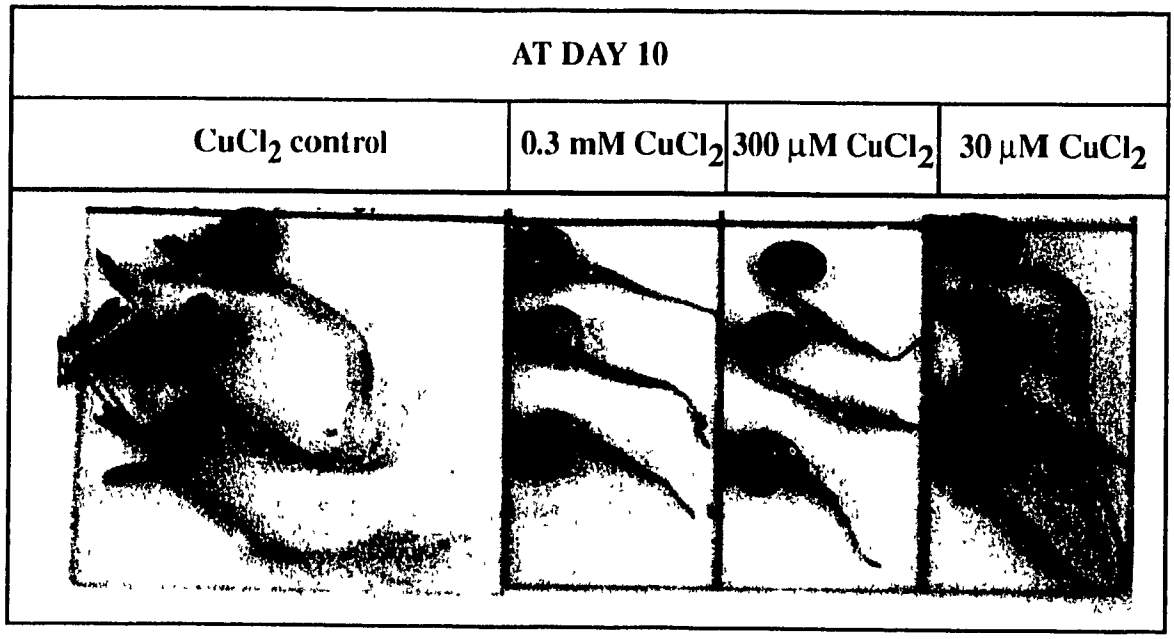


Figure 9: Continued

features were also observed with the chitosan controls. $CuCl_2$-treated seedlings did not develop lateral roots until day 10 as compared with the $CuCl_2$ control, except at the lowest concentration tested (e.g. 30 µM; Figure 9-D). In the latter case, however, lateral roots were very short. Tissue browning took place at all $CuCl_2$ concentrations tested (Figure 9-D). In all cases where tissue browning took place, the treated roots were difficult to homogenize (Figure 9), possibly due to lignification of these tissues. Examples of chromatographic fractionation are given in Figures 10 and 11 which show the HPLC profiles of extracts prepared from roots stressed with *Rhizobium lupini* and 300 µM $CuCl_2$, respectively. Figure 12 shows the HPLC profile of root exudates secured from *Rhizobium lupini*-stressed seedlings.

E.3.1. Elicitation with *Rhizobium lupini*

The variations in total isoflavonoids are reported for the root systems and the exudates of *Rhizobium lupini*-treated lupin seedlings as compared with the controls (Table 8A,B). At all sampled growth stages, the total isoflavonoids identified were lower in the root (two-fold at day 10) and two-fold higher in the exudates as compared with the controls. The decrease in total isoflavonoids identified in the stressed root system between days 7 and 10 was mostly at the expenses of glucosides, which correlated well with the dramatic increase in the amounts of mono- and diprenylated derivatives found in the exudates (Table 8-A,B). These increases varied from 2.3-fold for the aglycones and diprenyls, to 10-fold for the genistein monoprenyls in the exudates of 10-day old stressed roots. It is interesting to note the high relative abundances of genistein derivatives in root tissues and their exudates in both the controls and stressed tissues (Table 8-B), whereas those of exudates remained high for the treated tissues.

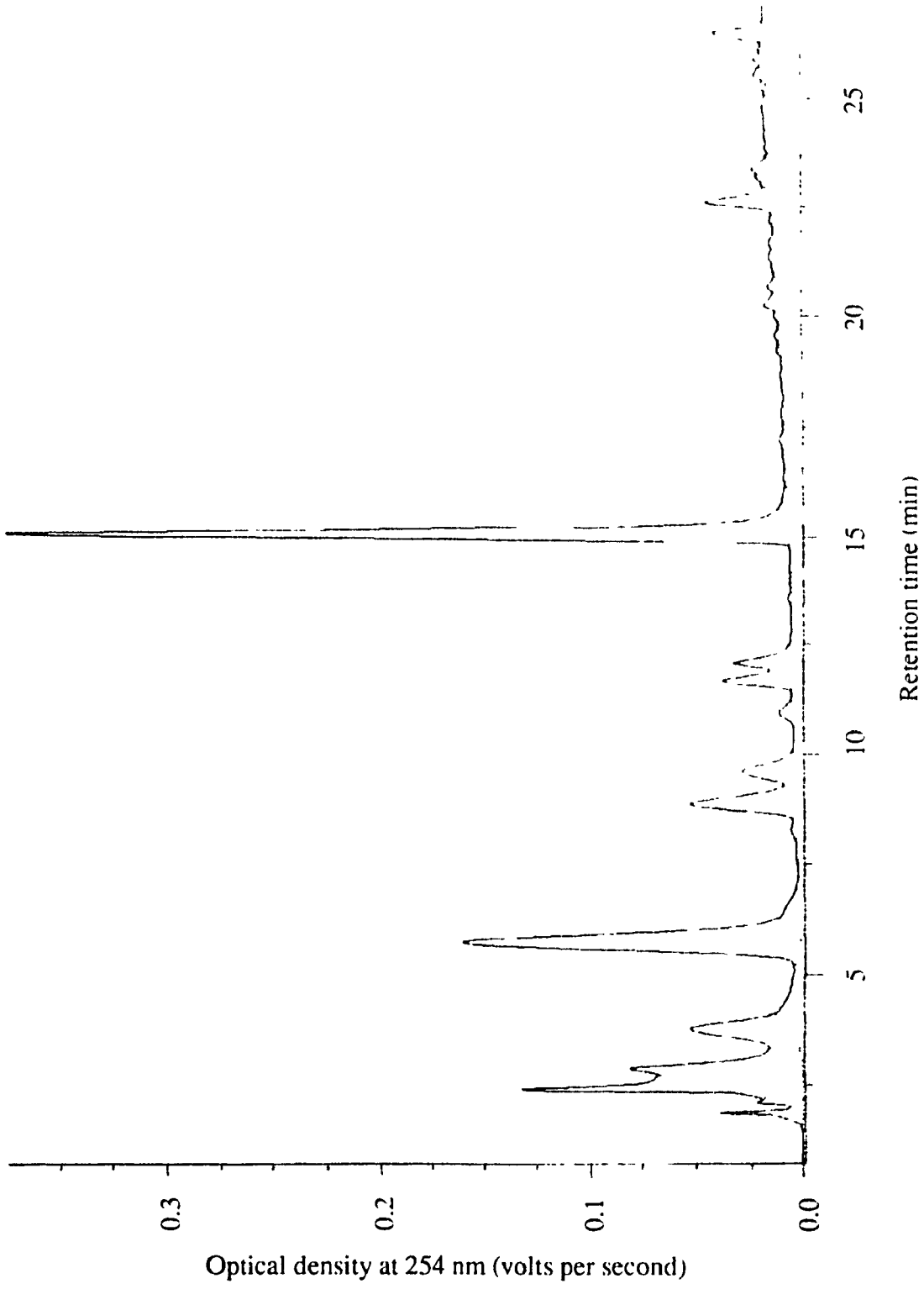


Figure 10: HPLC profile of 10 day-old root systems stressed with *Rhizobium lupini*

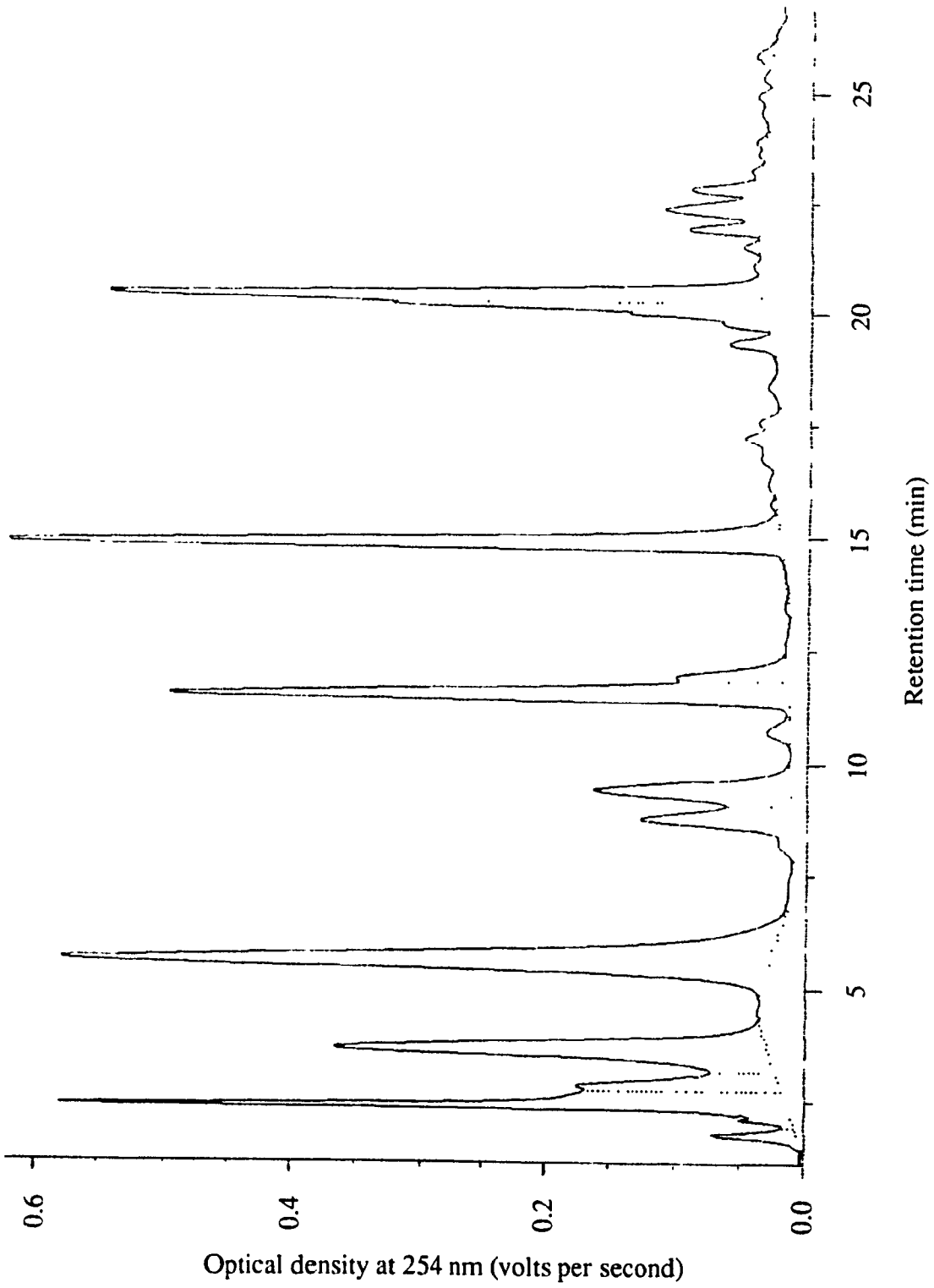


Figure 11: HPLC profile of 10 day-old root systems stressed with 300 μM $CuCl_2$

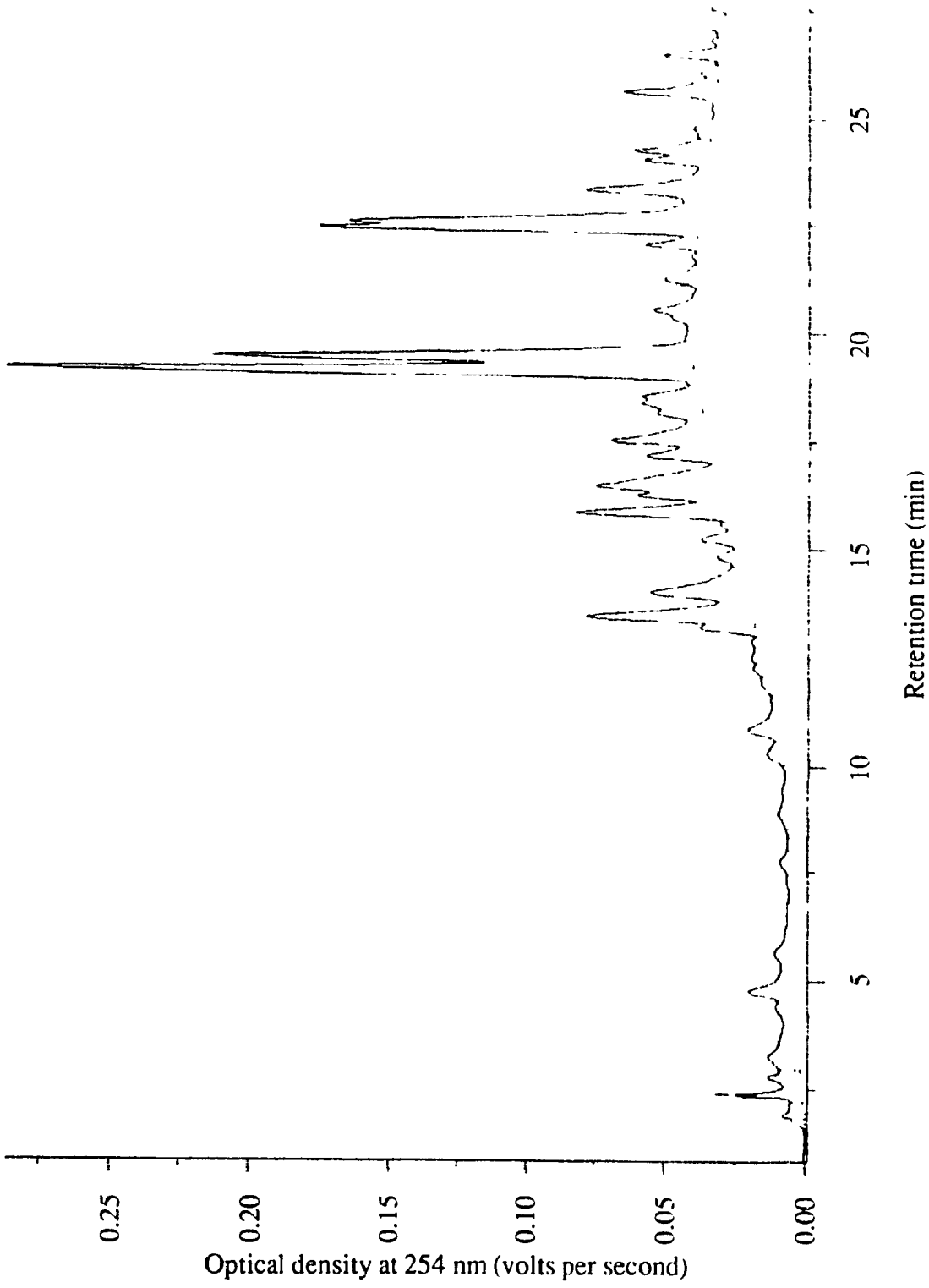


Figure 12: HPLC profile of exudates from root systems stressed with *Rhizobium lupini*

Table 8: Isoflavonoid content of lupin seedlings stressed with biotic elicitors[1]

	Roots			Exudates		
Isoflavonoid groups	4 D[2]	7 D	10 D	4 D	7 D	10 D
A: Control medium						
Glucosides	4.45[a]	3.88[e]	2.41[f]	0.21[f]	0.64[d]	0.82[f]
Aglycones	0.75[a]	0.43[f]	0.87[e]	0.08[b]	0.48[f]	2.90[e]
Gen. MonoPr.	0.01[c]	0.03[c]	0.07[c]	0.33[f]	0.76[d]	1.05[f]
2'-OH Gen. MonoPr.	0.01[f]	0.01[f]	0.03[f]	0.05[f]	0.15[f]	0.51[f]
Diprenyls	0.01[f]	0.00[b]	0.01[f]	0.06[f]	0.11[f]	0.14[c]
Total Identified[3]	5.22[a]	4.36[b]	3.43[c]	0.82[b]	2.29[b]	5.76[a]
Gen. Derivatives (%)	92.2[a]	85.6[c]	72.8[c]	63.3[c]	70.9[b]	35.1[e]
B: *Rhizobium lupini* suspension medium						
Glucosides	3.58[b]	3.31[b]	0.91[b]	0.38[f]	0.38[b]	0.47[f]
Aglycones	0.82[b]	0.41[b]	0.50[b]	0.20[f]	1.84[d]	1.65[a]
Gen. MonoPr.	0.05[b]	0.19[a]	0.17[b]	0.86[f]	7.30[f]	8.11[a]
2'-OH Gen. MonoPr.	0.01[f]	0.02[f]	0.03[f]	0.02[f]	0.36[e]	0.31[a]
Diprenyls	0.03[d]	0.06[a]	0.07[c]	0.03[f]	0.28[b]	0.49[d]
Total Identified	4.49[a]	4.00[b]	1.69[c]	1.75[d]	10.41[f]	11.02[a]
Gen. Derivatives (%)	85.4[a]	82.7[c]	77.3[c]	63.3[f]	80.4[f]	81.4[a]

.../continued

Table 8: Continued

	Roots			Exudates		
Isoflavonoid groups	4 D	7 D	10 D	4 D	7 D	10 D
C: Yeast extract medium						
Glucosides	3.73^{c}	3.2^{b}	2.69^{a}	3.66^{f}	2.24^{b}	2.95^{a}
Aglycones	1.56^{c}	1.06^{d}	0.75^{a}	3.76^{e}	4.1^{f}	12.2^{b}
Gen. MonoPr.	0.18^{c}	0.29^{c}	0.43^{a}	2.69^{f}	6.91^{f}	31.0^{a}
2'-OH Gen. MonoPr.	0.09^{d}	0.10^{c}	0.56^{c}	0.93^{e}	7.17^{f}	20.9^{f}
Diprenyls	0.03^{d}	0.03^{a}	0.03^{c}	0.12^{f}	0.28^{a}	1.73^{f}
Total Identified	5.69^{c}	4.84^{a}	5.25^{a}	12.31^{f}	22.04^{f}	71.79^{a}
Gen. Derivat. (%)	76.1^{c}	69.7^{a}	44.2^{b}	34.7^{f}	38.2^{f}	47.7^{a}
D: Chitosan control medium						
Glucosides	5.59^{a}	2.15^{f}	ND	1.91^{f}	2.31^{f}	ND
Aglycones	0.74^{a}	0.58^{f}	ND	5.67^{f}	13.17^{b}	ND
Gen. MonoPr.	0.02^{c}	0.02^{f}	ND	0.13^{f}	0.12^{e}	ND
2'-OH Gen. MonoPr.	0.01^{f}	0.03^{a}	ND	0.15^{f}	0.16^{f}	ND
Diprenyls	0.01^{f}	0.01^{b}	ND	0.01^{f}	0.07^{f}	ND
Total Identified	6.37^{a}	2.8^{e}	ND	7.95	16.05^{e}	ND
Gen. Derivatives (%)	92.6^{a}	82.9^{f}	ND	95.4^{f}	95.9^{d}	ND

.../continued

Table 8: Continued

	Roots			Exudates		
Isoflavonoid groups	4 D	7 D	10 D	4 D	7 D	10 D
E: Chitosan Medium						
Glucosides	6.02[b]	3.64[f]	ND	1.55[b]	5.35[f]	ND
Aglycones	1.04[a]	0.44[f]	ND	3.84[b]	25.29[a]	ND
Gen. MonoPr.	0.03[c]	0.20[c]	ND	0.33[f]	0.64[f]	ND
2'-OH Gen. MonoPr.	0.01[f]	0.25[a]	ND	0.09[c]	2.69[f]	ND
Diprenyls	0.01[f]	0.02[c]	ND	0.03[a]	0.20[f]	ND
Total Identified	7.17[b]	4.64[f]	ND	6.49[a]	41.50[c]	ND
Gen. Derivatives (%)	89.5[b]	75.7[f]	ND	83.3[b]	46.4[a]	ND

[1] Values are expressed in nmol/mg f.wt. for whole roots and nmol/seedling for exudates except for genistein derivatives (percent relative abundance); they represent averages of duplicate samples, with standard deviations indicating confidence levels of: a, <5%; b, <10%; c, <15%; d, <20%; e, <25%; f, >25%.

[2] Abbreviations: D, days; Gen., genistein; MonoPr., monoprenyls; ND, not determined (see Appendix B, for individual isoflavones included in each group).

[3] Totals include lupinalbin A (see Appendix B).

E.3.2. Elicitation with Yeast Extract

In contrast with *R. lupini*, elicitation with yeast extract resulted in dramatic increases (10-15-fold) of total isoflavonoids in the exudates and, to a lesser extent, in the root tissues, as compared with the controls (Table 8-A,C). In root tissue, these consisted mainly of 6- and 18-fold increases in the monoprenyl derivatives of genistein and 2'-hydroxygenistein at day 10, respectively (Table 8-C), as compared with the control (Table 8-A). The increases in exudate isoflavonoid groups ranged from 18 to 48-fold for 2'-hydroxygenistein monoprenyls, 8 to 30-fold for genistein monoprenyls and 2 to 12-fold for diprenyls (Table 8-C) as compared with the control (Table 8-A). Exudate aglycones and glucosides were 47 and 17-fold higher than the controls at day 4, respectively, and declined thereafter. The large amounts of isoflavonoids found in the exudates at all sampling dates indicate very active isoflavonoid synthesis in the seedling roots and their subsequent release into the medium in response to elicitation with yeast extract. Such elicitation resulted in a lower relative abundance of genistein derivatives in both roots and exudates at all stages of growth (except for 10 day-old exudates) as compared with those of the controls.

E.3.3. Elicitation with Chitosan

Chitosan treated-seedlings (0.005%) and their exudates were not analyzed at day 10 due to death of the tissues at this growth stage (Figure 9-C). In addition, secretion and accumulation were not reported for the other chitosan concentrations tested due to death of the seedlings at 0.05% chitosan, and because 0.0005% chitosan treatment did not exhibit any alterations in the different isoflavonoid compounds from the chitosan control. It should be noted that the buffered chitosan control medium (see Section D.2.4.) resulted in a 7-fold increase in total isoflavonoids in the exudates at the expense of the root systems (Table 8-D), as compared with those of a low-buffer capacity control (Table 8-A). These

exudates consisted mainly of aglycones (82% of total) and glucosides (14% of total), with small amounts of the other isoflavonoid groups (Table 8-D).

The total amount of isoflavonoids identified in both chitosan-treated root systems and their exudates were almost similar to those of the chitosan control at day 4 (Table 8-D,E). At 7 days, however, these increased by 2-fold in root systems and 2.6-fold in the exudates (Table 8-E) as compared with untreated seedlings (Table 8-D). In the root systems of 7 day-old treated seedlings, this increase was mostly due to a 10-fold rise in the amount of both monoprenylated derivatives. Major differences were also observed in the individual groups of compounds identified in the exudates at day 7. These consisted of 5- and 17-fold increases in genistein- and 2'-hydroxygenistein monoprenyls, respectively, as well as 2- to 3-fold increases for the other constituents (Table 8-E) as compared with the control exudates (Table 8-D). The relative abundance of genistein derivatives in 7 day old root systems and their exudates were reduced to 75.7% and 46.4%, respectively (Table 8-E), as compared with those of the control (Table 8-D).

E.3.4. Elicitation with Copper Chloride

Neither the seedlings treated with 3.0 mM $CuCl_2$ nor their exudates were analysed at day 10 due to severe tissue browning (Figure 9-D). The low pH of the $CuCl_2$ control medium (Table 9-A) resulted in slight increases in total isoflavonoids of the root systems and their exudates, as compared with those of the neutral-pH control. However, all of the isoflavonoids groups analyzed had almost similar relative abundance in these two controls (Tables 8-A and 9-A).

3.0 mM $CuCl_2$: Elicitation with 3.0 mM $CuCl_2$ resulted in a 2-fold increase in total root isoflavonoids, as well as increase of 18- and 7-fold in the exudates at days 4 and 7 (Table 9-B) as compared with the controls (Table 9-A). In the root system, the monoprenylated compounds constituted 16% of the total, with the 2'-hydroxygenistein monoprenyls

Table 9: Isoflavonoid content of lupin seedlings stressed with $CuCl_2$[1]

	Roots			Exudates		
Isoflavonoid groups	4 D[2]	7 D	10 D	4 D	7 D	10 D
A: CuCl2 control medium						
Glucosides	4.82[a]	4.98[a]	4.10[c]	0.15[f]	0.59[d]	3.59[b]
Aglycones	0.83[a]	0.46[f]	0.83[a]	0.08[f]	1.11[f]	0.55[f]
Gen. MonoPr.	0.02[f]	0.02[f]	0.05[b]	0.10[f]	1.36[b]	1.35[a]
2'-OH Gen. MonoPr.	0.00[e]	0.01[f]	0.02[d]	0.06[a]	0.84[b]	0.52[b]
Diprenyls	0.01[e]	0.01[e]	0.02[e]	0.08[f]	0.15[f]	0.22[e]
Total Identified[3]	5.69[a]	5.51[b]	5.02[c]	0.61[e]	4.96[b]	7.51[a]
Gen. derivatives (%)	91.8[a]	75.1[c]	73.9[c]	37.2[b]	50.1[b]	55.4[a]
B: CuCl2 (3.0 mM) medium						
Glucosides	6.10[c]	4.57[d]	ND	5.43[b]	9.61[b]	ND
Aglycones	2.15[d]	2.64[a]	ND	5.11[f]	11.74[e]	ND
Gen. MonoPr.	0.13[d]	0.20[f]	ND	0.14[f]	0.30[d]	ND
2'-OH Gen. MonoPr.	0.36[b]	1.14[a]	ND	0.02[a]	0.41[f]	ND
Diprenyls	0.01[e]	0.04[a]	ND	0.03[f]	0.06[a]	ND
Total Identified	8.83[c]	8.88[b]	ND	11.10[d]	34.94[b]	ND
Gen. derivatives (%)	76.9[c]	31.3[f]	ND	89.6[e]	51.7[c]	ND

.../continued

Table 9: Continued

Isoflavonoid groups	Roots 4 D	Roots 7 D	Roots 10 D	Exudates 4 D	Exudates 7 D	Exudates 10 D
C: $CuCl_2$ (300 µM) medium						
Glucosides	5.27^{a}	4.73^{a}	4.62^{e}	1.85^{f}	5.61^{b}	3.78^{a}
Aglycones	1.63^{b}	0.80^{a}	1.82^{a}	7.05^{f}	23.63^{f}	27.85^{d}
Gen. MonoPr.	0.22^{b}	0.27^{a}	0.88^{d}	0.70^{a}	3.83^{d}	7.27^{f}
2'-OH Gen. MonoPr.	0.42^{b}	0.98^{f}	1.56^{b}	2.22^{a}	10.38^{f}	14.94^{f}
Diprenyls	0.02^{f}	0.03^{e}	0.09^{a}	0.05^{f}	0.03^{e}	0.31^{f}
Total Identified	7.61^{a}	7.0^{b}	9.48^{e}	30.60^{e}	59.15^{f}	60.26^{f}
Gen. derivatives (%)	23.3^{a}	45.9^{a}	51.4^{d}	18.5^{f}	24.0^{e}	28.1^{f}
D: $CuCl_2$ (30 µM) Medium						
Glucosides	4.91^{a}	4.12^{b}	3.96^{f}	0.37^{f}	0.39^{f}	0.64^{e}
Aglycones	1.27^{e}	0.62^{b}	1.58^{f}	9.09^{f}	14.84^{f}	13.95^{b}
Gen. MonoPr.	0.07^{e}	0.09^{f}	0.38^{f}	4.57^{a}	7.97^{f}	16.57^{a}
2'-OH Gen. MonoPr.	0.10^{f}	0.14^{f}	0.71^{f}	7.59^{a}	9.67^{f}	22.67^{b}
Diprenyls	0.01^{d}	0.02^{a}	0.03^{b}	0.06^{f}	0.22^{f}	0.38^{e}
Total Identified	6.37^{a}	5.05^{b}	7.12^{f}	22.99^{e}	36.63^{f}	64.67^{b}
Gen. derivatives (%)	87.5^{b}	70.0^{a}	57.2^{e}	28.7^{f}	28.6^{f}	37.7^{b}

$^{1-3}$, as in Table 8.

reaching a 114-fold increase at day 7 (Table 9-B) as compared with the control (Table 9-A). These results suggest an active synthesis of monoprenylated isoflavonoids in $CuCl_2$-stressed seedlings. In the exudates, the increases in isoflavonoids were most prominent for the aglycones (64-fold) and the glucosides (36-fold) at day 4. This represents the first observation where both aglycones and glucosides amount to ca 60% of all identifed isoflavonoids in the exudates, and may be due to their 'leaching' from damaged root cells at such high $CuCl_2$ concentration.

However, the relative abundance of genistein derivatives in $CuCl_2$-stressed roots (Table 9-B) were remarkably lower than those of the control (Table 9-A), especially at day 7, although they were much higher for the exudates.

300 μM $CuCl_2$: As compared with a <2-fold increase in total isoflavonoids in root tissue, elicitation with 300 μM $CuCl_2$ resulted in a dramatic increase in root exudates during all stages of growth (Table 9-C) as compared with untreated tissues (Table 9-A). These increases amounted to 50-, 12- and 8-fold by days 4, 7 and 10, respectively. The most significant increase in exudate constituents are accounted for mostly by extremely high levels of aglycones (50 to 80-fold), 2'-hydroxygenistein monoprenyls (12 to 37-fold) and genistein monoprenyls (2.5 to 7-fold) as compared with the correseponding controls. Root isoflavonoids exhibited a 10 to 18-fold increase in genistein monoprenyls and a more dramatic 40 to 78-fold increase in 2'-hydroxygenistein monoprenyls, at the expense of glucosides at day 10 (Table 9-C). Interestingly, there was a noticable decrease in the relative abundance of genistein derivatives which amounted to <52% in the roots and <28% in the exudates, during all the growth stages.

30μ M $CuCl_2$: The results of this treatment are quite similar to those of 300 μM $CuCl_2$ especially with regards to the dramatic increase in the total isoflavonoids identified in the exudates, and to the marked accumulation and secretion of aglycones and monoprenylated

derivatives (Table 9-D). However, the relative abundance of genistein derivatives was higher at all growth stages in these stressed roots (Table 9-D) as compared with the other $CuCl_2$ concentrations (Table 9-B, C). In addition, the increased levels of both monoprenyl groups (at the expense of glucosides) in seedling exudates resembles more those of the control (Table 9-A) than with other $CuCl_2$ concentrations. These results suggest an elicited isoflavonoid secretion in response to a 30μ M $CuCl_2$ concentration, as opposed to their leaching caused by root cell damage which may have resulted from treatments with higher concentrations.

In summary, it appears that all of the $CuCl_2$ concentrations tested resulted in a significant increase in isoflavonoid secretion, and in a remarkable increase in the relative abundance of aglycones and monoprenyls (mostly derived from 2'-hydroxygenistein). However, the increased levels of glucosides observed in the exudates of seedlings treated with high $CuCl_2$ concentrations seem to result from a disrupted secretion-pattern in response to both elicitation and cell-membrane damage, whereas 30μM $CuCl_2$ resulted in elicitation only, since glucoside levels were similar to those of the control. Furthermore, a dose-response is observed with $CuCl_2$ elicitation, as revealed by the increased levels of 2'-hydroxygenistein monoprenyls and total isoflavonoids (Table 9).

E.4. ISOFLAVONOID COMPOSITION OF LATERAL ROOTS AND NODULES

The isoflavonoid composition of nodules secured from 6 wk-old plants and of the lateral roots harboring them were determined (Figures 13 and 14), and compared with 2 wk-old uninfected lateral roots (Table 10; for experimental design see Section C.6.4.).

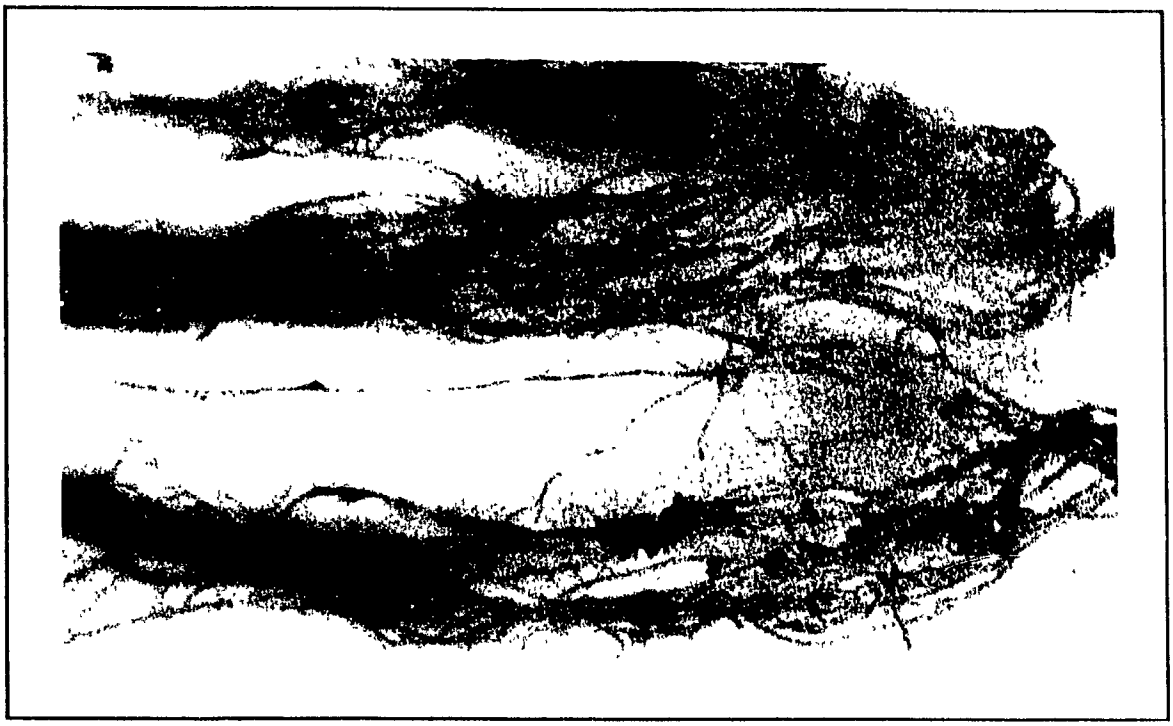


Figure 13: Root systems harboring nodules: Arrows indicate large- and small sized nodules, >4 mm and <2 mm diameter, respectively.

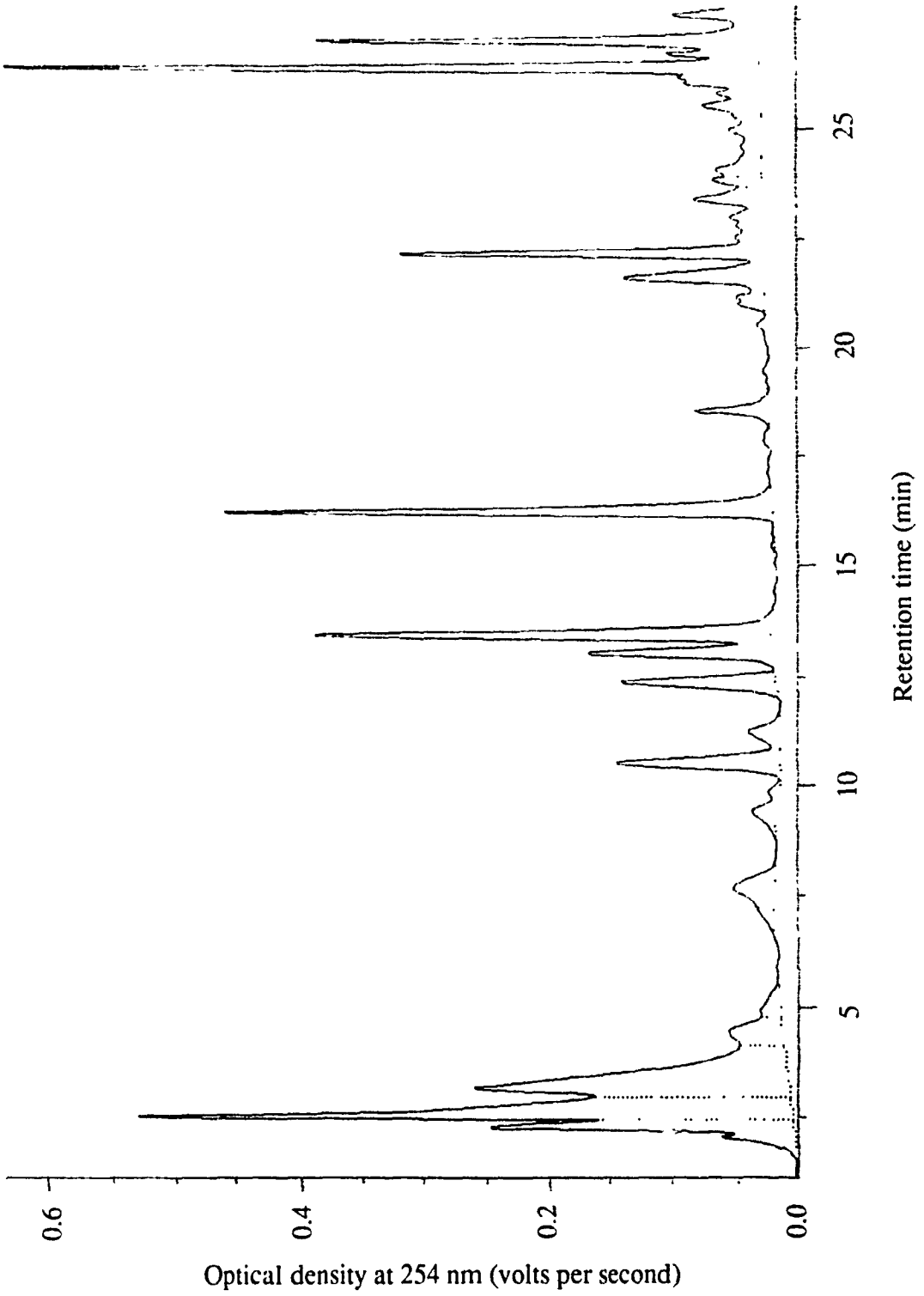


Figure 14: HPLC profile of 2 to 4 mm nodules

Table 10: Isoflavonoid composition of lateral roots and nodules[1]

	Lateral roots		6 Week-old nodules		
Isoflavonoid groups	2 Weeks	6 Weeks	0-2 mm	2-4 mm	>4 mm
Glucosides	1.51^{b}	0.69^{a}	0.89^{a}	0.21^{e}	0.54^{b}
Aglycones	0.12^{a}	3.42^{a}	1.87^{c}	0.66^{b}	0.92^{f}
Gen. MonoPr.[2]	0.00^{d}	0.40^{a}	1.30^{d}	0.76^{e}	0.54^{b}
2'-OH Gen. MonoPr.	0.03^{c}	0.16^{b}	1.43^{d}	0.45^{f}	0.39^{b}
Lupalbigenin	0.03^{a}	0.33^{b}	1.40^{c}	0.98^{c}	0.63^{c}
2'-OH lupalbigenin	0.00^{a}	0.13^{a}	2.19^{e}	1.41^{c}	1.39^{c}
Total identified[3]	1.77^{b}	5.60^{a}	9.54^{a}	4.63^{c}	4.55^{a}
Gen. derivatives (%)	77.3^{b}	56.9^{a}	40.4^{a}	49.4^{c}	45.4^{c}

[1]Values are expressed in nmol/ mg f. wt. except for genistein derivatives (percent relative abundance); they represent averages of duplicate samples, with standard deviations indicating confidence levels of: a, <5%; b, <10%; c, <15%; d, <20%; e, <25%; f, >25%.

[2]Abbreviations: D, days; Gen., genistein; MonoPr., monoprenyls (see Appendix B, for individual isoflavones included in each group).

[3]Totals include lupinalbin A (see Appendix B).

E.4.1. Isoflavonoid Content of Lateral Roots

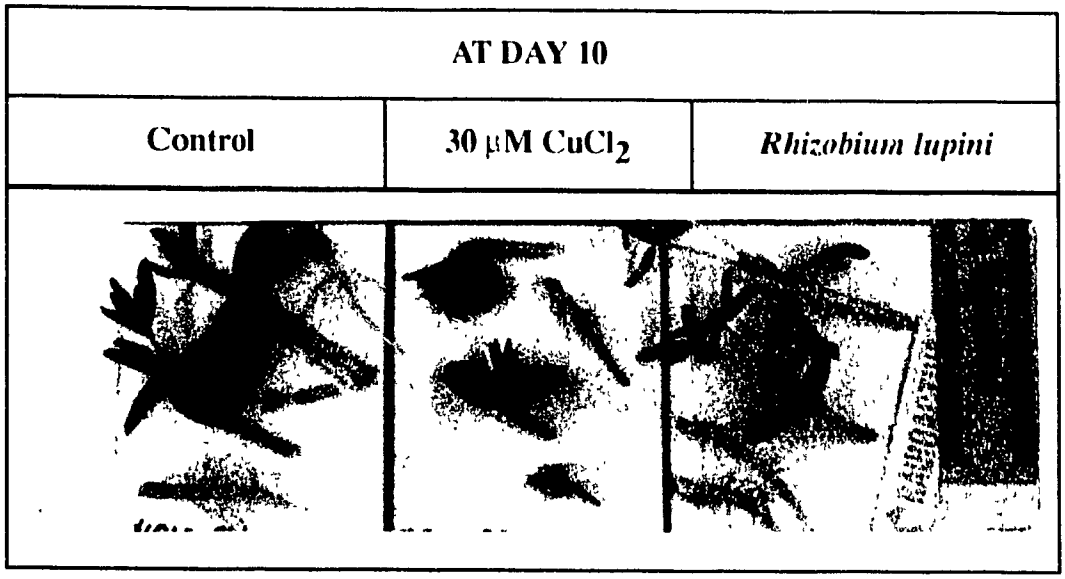
During growth of lateral roots there was a decrease in the amount of glucosides with a corresponding increase in the algycones and, to a lower extent, of all the prenylated compounds. However, these changes were accentuated by a 3-fold net increase in the total isoflavonoids identified, during the 4-wk growth period, resulting in the highest level (61% of total) so far observed for aglycones in the root tissue. The relatively high abundance of genistein derivatives in 2-wk old roots suggests a low 2'-hydroxylating activity in this tissue, as compared with the 6 wk-old roots (Table 10).

E.4.2. Isoflavonoid Content of Nodules

The results shown in Table 10 clearly demonstrate the presence of higher amounts of isoflavonoids in the small size nodules than in the intermediate- or large-sized nodules. This decrease in isoflavonoid content during maturation of nodules may be due to an increased secretion into the medium. This seems plausible, considering the dramatic increase in isoflavonoid content of root exudates, especially under stress conditions (Tables 8 and 9). The relative abundance of isoflavonoid groups was quite similar for all size nodules, except for the diprenyls whose relative abundance was the highest reported so far (ca 38 to 52%). Especially prominent in the nodule tissues was the high level of 2'-hydroxylupalbigenin which constituted >22% of total isoflavonoids at all maturation stages. As compared with lateral roots, the relative low abundance of genistein derivatives in the nodules may be the result of a stress-induced 2'-hydroxylase activity caused by the symbiotic association.

E.5. BIOSYNTHESIS OF ISOFLAVONOIDS IN SEEDLINGS

Lupin seedlings (Figure 15) were administered radiolabeled *t*-cinnamic acid under different growth conditions (Appendix D). The incorporation of label into major groups of



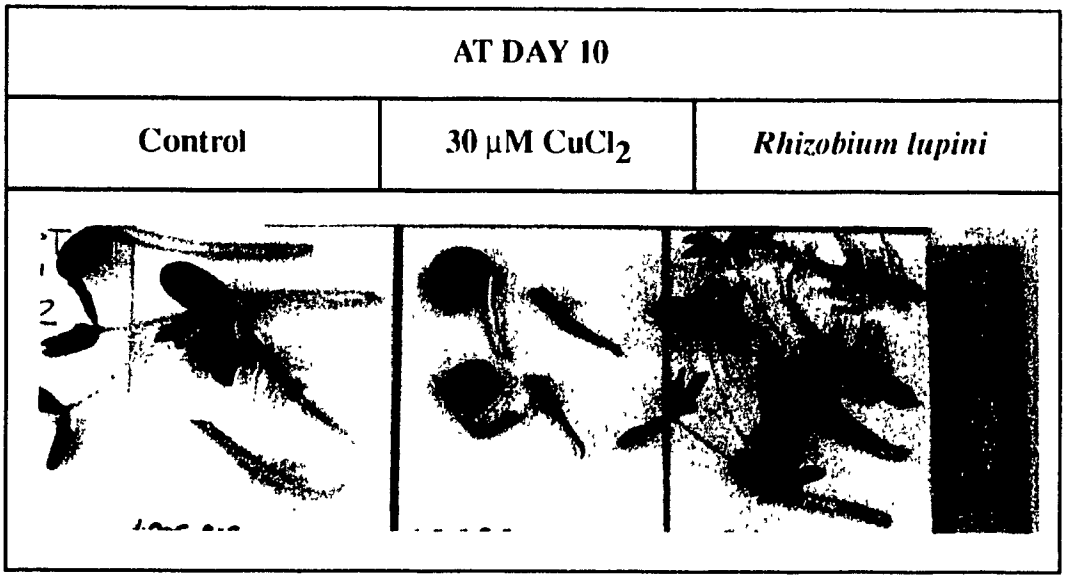


Figure 15: Photographs of lupin seedlings utilized for feeding experiments

isoflavonoids was determined by HPLC fractionation of extracts from each treatment (Tables 11 and 12). Two treatments, A and B (Table 11), were pulsed for 48 and 24 hrs and harvested at days 4 and 3, respectively. Three others treatments were pulsed for 24 hrs, further chased for 7 days (treatments C, D and E, Table 12) then harvested at day 10. The isoflavonoid constituents of the root soluble fractions were analysed by HPLC for all treatments. In addition, the isoflavonoids of the cotyledons soluble fraction were determined for pulse labeling studies (treatments A and B), whereas those of seedling exudates were determined in pulse-chase experiments (treatments C, D and E).

The results of treatments A and C represent the biosynthesis of isoflavonoids in intact control seedlings at days 4 and 10, respectively. Those of treatment B represent pulse labeling of excised segments (roots and cotyledons) and should determine the capability of these organs for isoflavonoid biosythesis, as compared with the control (treatment A) where translocation of metabolites from root to cotyledons may occur. Treatments D and E were chased in $CuCl_2$ solution, or in *R. lupini* suspension, respectively, in order to study the effect of stress conditions on the steady state biosynthesis of isoflavonoids (Table 12).

E.5.1. Pulse Labeling Studies with Intact and Excised Seedlings

The total incorporation of label into the intact seedlings (treatment A) appears to be 3.3-fold higher than that of the excised segments (treatment B, Table 11), indicating a higher label uptake when feeding is performed with the intact root, compared with excised segments. The ratio of radioactivity found in cotyledons to that in the root system in the control seedlings is 1:15 (column A, Table 11). This ratio is almost similar to that of the pulse labeling of excised seedlings (ca 1:13, treatment B).

The cinnamate label was incorporated into glucosides, aglycones and prenylated compounds of the root system in a ratio of 93:6:1, respectively (treatment A, Table 11), whereas this ratio changed to 72:25:3 in the case of cotyledons. Roots, therefore, accumulate more glucosides and less of the aglycones and prenylated compounds than the

Table 11: Pulse incorporation studies with a flavonoid precursor[1]

	Roots		Cotyledons	
Isoflavonoid groups[2]	A[3]	B	A	B
Glucosides	92.6	80.3	72.5	46.1
Aglycones	5.8	18.0	24.6	50.2
Gen. MonoPr.[4]	0.5	0.4	0.8	0.2
2'-OH Gen. MonoPr.	0.4	0.6	0.5	1.9
Diprenyls	0.1	0.1	1.3	0.6
Total (% of label fed)[5]	20.39	6.09	1.30	0.46
Gen. Derivatives	52.4	89.1	53.6	56.2

[1]Seedlings were pulsed with [2-^{14}C] cinnamic acid; the cotyledons and the root system were extracted and analyzed by HPLC at the end of the metabolic period. Values shown (except totals, see below) represent the incorporation of label in identified isoflavonoid fractions, relative to the total radioactivity in all identified fractions.

[2]See Appendix B for individual isoflavones included in each group.

[3]A and B denote different treatments; A, control pulse for 48 hr, 1.0 μCi of cinnamic acid; B, excised seedling pulse for 24 hr, 0.625 μCi of cinnamic acid, as described in Appendix D.

[4]Abbreviations: Gen., genistein, MonoPr., monoprenyls.

[5]Values shown represent the relative incorporation of label (% of label fed, Appendix D) into isoflavonoids identified. Totals include lupinalbin A (Appendix B).

Table 12: Pulse-chase incorporation studies with a flavonoid precursor[1]

	Roots			Exudates		
Isoflavonoid groups[2]	C H_2O	D $CuCl_2$	E *R. lup.*	C H_2O	D $CuCl_2$	E *R. lup.*
Glucosides	46.5[b]	33.2[a]	40.2[a]	15.6[b]	5.1[a]	5.5[b]
Aglycones	38.5[a]	33.8[c]	51.3[a]	40.9[a]	29.0[b]	21.5[a]
Gen. MonoPr.[3]	5.0[c]	8.4[a]	3.1[f]	19.3[b]	28.3[b]	35.6[b]
2'-OH Gen. MonoPr.	5.7[a]	17.0[e]	3.5[e]	13.1[a]	24.9[a]	23.2[c]
Diprenyls	3.1[b]	5.8[c]	1.5[d]	4.9[a]	3.9[a]	7.5[d]
Total (% of label fed)[4]	5.87[a]	2.50[c]	18.63[a]	0.65[d]	1.92[a]	1.54[b]
Gen. Derivatives	56.5[c]	47.1[a]	48.8[a]	50.2[a]	50.5[b]	56.9[b]

[1]Seedlings were pulsed for 24 hr with 0.25 µCi of [2-^{14}C] cinnamic acid, and chased for 7 days in water (D), 30 µM $CuCl_2$ solution (E) or in a *R. lupini* suspension (F). At the end of the metabolic period, root systems and their exudates were analyzed by HPLC. Values shown (except totals) represent the incorporation of label in identified isoflavonoid fractions, relative to the total radioactivity in all identified fractions. Superscript letters refer to standard deviations, with confidence levels of: a, <5%; b, <10%; c, <15%; d, <20%; e, <25%; f, >25%.

[2]See Appendix B for individual isoflavones included in each group.

[3]Abbreviations: Gen., genistein, MonoPr., monoprenyls.

[4]Values shown represent the relative incorporation of label (% of label fed, Appendix D) into isoflavonoids identified. Totals include lupinalbin A (Appendix B).

cotyledons. However, genistein derivatives constituted 53.0% of the total isoflavonoid pool of both organs analyzed (Table 11). These results indicate the capability of *L. albus* seedlings for the biosynthesis of the different groups of isoflavonoids.

Incorporation of the cinnamate label into isoflavonoids of excised segments (treatment B, Table 11), resulted in a different incorporation ratio into the glucosides and aglycones, of both the roots (80:18) and the cotyledons (46:50), as compared with those of intact seedlings (treatment A). These results demonstrate the capability of both the excised radicle and cotyledons for the biosynthesis of isoflavonoids. However, it is not known whether the variations in the ratios of glucosides to aglycones, in the intact seedling and the excised roots and cotyledons, are due to a higher ß-glucosidase activity, or a higher aglycone synthesis in the excised tissues resulting from segment excision. Nonetheless, these effects may be attributed to a wound response. In addition, excised roots tend to synthesize more genistein-derived compounds as compared with those of intact seedlings. The predominant synthesis of genistein-derived compounds in excised root tissues, suggests the translocation of 2'-hydroxygenistein-derived compounds from the cotyledons and/or a low 2'-hydroxylase activity in the root tissue.

The results obtained with both treatments (A and B, Table 11) indicate the existence, in this tissue, of the enzyme complement required for isoflavonoid synthesis, including chalcone synthase and isoflavone synthase, as well as the glucosyl- and prenyl-transferases. Labeling experiments with L-[U-^{14}C]-phenylalanine (data not shown) resulted in the labeling isoflavonoids in a manner similar to that of treatment B, indicating an active phenylalanine ammonia-lyase (PAL) activity in these tissues. However, some differences in isoflavonoid composition of excised seedling parts were observed when phenylalanine was utilized as the radiolabeled precursor as compared with cinnamic acid.

E.5.2. Pulse-Chase Labeling Studies with Intact Seedlings

The total incorporation of label into root and exudate isoflavonoids of the control- and $CuCl_2$-chase (treatments C and D, respectively, Table 12) is almost similar, whereas it is 3-fold as high for seedlings chased with *R. lupini* (treatment E). This difference may be due to the fact that the stress caused by *R. lupini* may lead to an increased isoflavonoid biosynthesis in the seedling. However, the latter possibility cannot explain this difference since the pulse incorporation was performed prior to chase and elicitation. Otherwise, some variations among the isoflavonoid pools may account for these differences in label incorporation. It is interesting to note that using $CuCl_2$ solution or *R. lupini* suspension as chase media resulted in an altered ratio of label incorporation into the isoflavonoids of the seedling root to that of their exudates. These ratios amounted to 1.3:1 in the $CuCl_2$- and 12:1 in *R. lupini*-chase, as compared with 8:1 in the water-chase control (Table 12). These results indicate that a water- or *R. lupini* -chase resulted in almost similar ratios (i.e. lower isoflavonoid secretion in exudate), whereas a chase in a $CuCl_2$ solution resulted in a higher secretion of labeled metabolites in the exudates (at the expense of that found in the root systems), as compared with those of the control (C).

E.5.2.1. Non-Elicited Controls

The cinnamate label incorporation into the root system, was found mainly in the glucosides and aglycones, with the remainder being incorporated into genistein- and 2'-hydroxygenistein-monoprenyls, and diprenyls in a ratio of **46 : 38 : 5 : 5 : 3**, respectively (treatment C, Table 12), as compared with a respective ratio of **16 : 41 : 19 : 13 : 5** in the exudates. These results suggest that prenylated isoflavonoids are more abundant in the exudates as compared with the root tissues, mostly at the expense of glucosides (treatment C, Table 12).

The genistein derivatives constituted ca 53% of the isoflavonoid compounds in both the roots and their exudates (treatment C, Table 12); these levels are similar to those observed

in pulse-labeled roots of the intact seedling (treatment A, Table 11). However, the steady state biosynthesis of isoflavonoids in control seedling roots at day 10 (treatment C, Table 12) resulted in higher levels of labeled-aglycones with a corresponding decrease in labeled glucosides, as compared with the 4 day-old pulsed controls (treatment A, Table 11). In addition, the fact that the label traced in the cotyledons at day 10 was relatively low (data not shown), suggests that isoflavonoids may be translocated to other parts of the seedling at later stages of germination.

E.5.2.2. Elicitation with Copper Chloride Solution

Approximately 33% of the cinnamate label was incorporated into each of the glucosides and the aglycones, of the soluble fraction of $CuCl_2$-treated roots, whereas the rest of label was incorporated into genistein monoprenyls, 2'-hydroxygenistein monoprenyls and diprenyls in a ratio of **33 : 34 : 9 : 17 : 6**, respectively (treatment D, Table 13). These results differ from those of the control (treatment C) in that the levels of labeled glucosides are significantly reduced in the $CuCl_2$-treated seedlings, and corroborate with the increased synthesis of prenylated compounds, especially the 2'-hydroxygenistein monoprenyls. In the exudates, however, the respective incorporation ratio is **5 : 29 : 28 : 25:4** (Table 12), suggesting higher rates of biosynthesis and secretion of both monoprenyl groups as compared with the control (treatment C). Elicitation with $CuCl_2$, therefore, leads to an increased synthesis of the 2'-hydroxygenistein monoprenyls in the roots, and the release of both monoprenyl groups in the exudates. This is further illustrated by the lower ratio of genistein derivatives in the roots as compared with that of the control (C).

E.5.2.3. Elicitation with *Rhizobium lupini* Suspension

The cinnamate label was almost equally incorporated into both the glucosides and aglycones in a ratio of 40:51 for the root system (treatment E, Table 12), whereas the prenylated compounds were relatively less abundant than in the control tissue (treatment C,

Table 12). However, 35.6% of the label was incorporated into the genistein monoprenyls in the exudates, which was 2-fold higher than the control. Similarly, other prenylated compounds were higher in the exudates of *R. lupini*-treated seedlings as compared with the control, whereas the glucosides and aglycones were reduced by 3- and 2.5-fold, respectively. Genistein derived isoflavonoids were slightly higher in these exudates, as compared with the control (ca 57 to 50%, respectively). These results suggest that the chase with *R. lupini* suspension results in decreased level of prenylation in the roots, whereas the composition of exudates exhibits higher levels of prenylated compounds, especially the genistein monoprenyls.

E.6. EFFECT OF FLAVONOIDS AND CINNAMIC ACID ON BACTERIAL GROWTH RATE

The effects of three different concentrations of cinnamic acid and of several flavonoids on the growth of five bacterial strains were studied. The data (Appendix E) is presented as relative growth rates for these treatments since (a) bacterial species grow naturally at different rates, (b) not all of the test compounds are soluble in the same solvent, and (c) the assays were performed at different temperatures depending on the bacterial species used (see Section D.7.). In Appendix E, a relative growth rate >1.0 denotes an inhibition of growth, as opposed to those having a value <1.0 which denotes a stimulation of growth. The detailed results of ANOVA tests are presented in Appendix F. In addition, a Student-Newman-Keuls test was performed for the highest concentrations tested in order to rank the means of each treatment (Table 13).

The relative growth rates for the various treatments of *E. coli* were all significantly different from the control (Appendix E; at P=(.995), Appendix F). All compounds tested resulted in an increased growth of *E. coli* , with genistein being the least effective, and quercetin the most effective (Table 13); sample curves of *E. coli* are shown in Appendix G.

Table 13: Student-Newman-Keuls ranking for bacterial growth rates in liquid medium[1]

Escherichia coli	*Bacillus subtilis*	*Agrobacterium rhizogenes*	*Rhizobium meliloti*	*Rhizobium lupini*
Control	2'-OH-Lupal.[2]	2'-OH-Lupal.	Luteone	2'-OH-Lupal.
Genistein	Derrone	Luteone	Lupinalbin A	Luteone
2'-OH-Gen.	Control	Lupinalbin A	Control	Lupalbigenin
Lico. A	Quercetin	Wighteone		Wighteone
Lupalbigenin		Genistein		Lico. A
2'-OH-Lupal.		2'-OH-Gen.		Control
Lupinalbin A		Lupalbigenin		Quercetin
Luteolin		Lico. A		
Luteone		Quercetin		
Derrone		Control		
Wighteone				
Cinnamic acid				
Quercetin				

[1]Composite ranking of compounds assayed at 10 μM (see Appendix E for other concentrations tested) whose effects significantly differ from the controls with ANOVA tests (see Appendix F for confidence levels). Compounds are listed starting from the most inhibitory (above the control) to the most stimulatory (below the control) for the growth of these bacteria in liquid medium; the control being the boundary between growth inhibition and stimulation. Shaded areas comprise homogeneous subsets (compound(s) having similar effects) whose effects differs from other subsets and controls. Darker shades denote the more effective subsets, as compared with other lighty-shaded subsets. Dash-outlined compounds (for *A. rhizogenes*) are not significantly different from the control, although growth seems to be affected (see growth curves in Appendices I, J and K); they are, therefore, ranked according to relative growth rate (see Appendix E). Outlined quercetin subsets are significantly different but are not comparable to other subsets, due to its solubility.

[2]Abbreviations: Gen., genistein; Lupal., lupalbigenin; Lico. A, licoisoflavone A.

There was no correlation observed between the presence or absence of prenyl groups on the compounds tested and the extent of their effect on growth rates.

Three compounds tested at 10 μM were found to affect significantly the growth rates of *B. subtilis* (Appendix E); these are quercetin, derrone and 2'-hydroxylupalbigenin, (P=(0.9948), P=(0.99995) and P=(0.99995), respectively, Appendix F). A correlation seems to exist between the presence of prenyl groups on the compounds tested and their inhibitory activity since quercetin stimulates growth whereas derrone and 2'-hydroxylupalbigenin inhibit growth (Table 13). Sample curves of *B. subtilis* are shown in Appendix H.

Two compounds tested at 10 μM were found to affect significantly the growth rates of *A. rhizogenes* (Table 13). These were quercetin and 2'-hydroxylupalbigenin (P=(0.9999) and P=(0.99995), respectively, Appendix F). However, the growth rates of *A. rhizogenes* seemed to be affected, in a decreasing order, by 10 μM of either luteone, lupalbin A, wighteone, genistein, 2'-hydroxygenistein, lupalbigenin, or licoisoflavone A (Table 13). When these compounds were tested, the growth curves (Appendices H, I, J and K) exhibited a behavior suggesting a transient growth inhibition (i.e. the slopes were not linear) which may have obscured the expected differences. However, a correlation seems to exist between the presence of prenyl groups on the compounds tested and their inhibitory activity (Table 13).

Two compounds tested at 10 μM were found to affect significantly the growth rates of *R. meliloti* (Table 13). These were luteone and lupinalbin A (P=(0.99995), Appendix F) and sample curves are shown in Appendix L. No correlation was observed between the presence of prenyl groups on the compounds tested and their inhibitory activity (Table 13).

Except derrone, all the prenylated compounds tested were found to affect significantly the growth rates of *R. lupini* (Table 13). These were, in a decreasing order of magnitude, 2'-hydroxylupalbigenin, luteone, lupalbigenin, wighteone, and licoisoflavone A (P=(0.99995, Appendix F). The growth curves obtained with *R. lupini* at 10 μM are

shown in Appendices M, N and O, together with that of derrone as an example of a non-effective prenylated coumaronochromone. Interestingly, none of the isoflavonoid aglycones tested on *R. lupini* affected significantly its relative growth rate (Table 13, Appendix O), except for quercetin which resulted in a significant increase in growth rate at all concentrations tested (Appendix E; P=(0.999), Appendix F). A correlation seems to exsist between the presence of prenyl groups on the compounds tested and their inhibitory activity (Table 13).

The results obtained with *E. coli* are as expected, since being an enteric bacteria it must have adapted to thrive on plant phenolics which are present in the diet. Compared with *E coli*, *B. subtilis* lives in soils, and may be suceptible to a few compounds as observed in the results (Table 13). In addition, a correlation between prenylation and growth inhibition seems to take place. A similar correlation is observed with *A. rhizogenes*, and such results are expected since *A. rhizogenes* is a plant pathogen, where prenylated compounds should be effective as phytoalexins on this species.

The results obtained with *R. lupini* indicate that prenylation of isoflavonoids also correlates with their inhibitory effect. These results indicate that diprenyls are more inhibitory than monoprenyls, given that 2'-hydroxylation leads to an additional decrease of growth rate (except for licoisoflavone A; Table 13). Therefore, lupin isoflavonoids affect *R. lupini* growth rate in a specific manner. It may be noted that the biotransformation of quercetin was shown to take place in this bacteria (Rao *et al*, 1991), and our results indicate a significant increase of growth rate in the presence of quercetin, which supports the published data. In contrast with *R. lupini*, both the diprenylated isoflavonoids assayed did not affect *R. meliloti* growth, whereas an aglycone and a monoprenylated compound did, thus indicating that such compounds exert a species-specific inhibitory effect on bacterial growth. Since prenylation does not appear to affect *R. meliloti.*, whereas it does for *R lupini*, these results illustrate the inability of the former bacteria to infect white lupin and demonstrate the specificity of their action on the lupin symbiont.

F. DISCUSSION

White lupin lends itself well to the study of isoflavonoids because of the wide structural diversity of compounds found in its tissues and, in contrast with other legumes, it constitutively expresses the prenylation of isoflavonoids. In addition to the well known role of substituted and lipophilic derivatives as phytoalexins (Lamb *et al*, 1989), flavonoid aglycones have been shown to act as molecular determinants of host specificity in symbiotic interactions of the Leguminosae with *Rhizobium* species (Mulligan and Long, 1985; Peters *et al.*, 1986). They were also shown to act as signal molecules in the development of both mono- and dicotyledonous plants (Mo *et al.*, 1992; Ylstra *et al.*, 1992). However, their glucosylated derivatives were reported to have no specific role in development (Mo *et al.*, 1992), and have a much lower *nod*-gene inducing activity than the corresponding aglycones (Hartwig and Phillips, 1991; Phillips, 1992). In addition, several studies have demonstrated the involvment of host phytoalexins in both the control of *Rhizobium* infection and the arrest of symbiosis (e.g. nodule senescence) in the Leguminosae. Hereafter, we shall refer to these molecules as 'symbiotic phytoalexins'. However, the latter have only been reported in a few cases, such as alfalfa (Dakora *et al.*, 1993) and soybean (Schmidt *et al.*, 1992; Karr *et al.*, 1992). Analysis of the isoflavonoid constituents in germinating lupin seeds presented in this study may help understanding of the role(s) of these compounds in this plant. Furthermore, pulse-chase labeling experiments were conducted with lupin seedlings to further corroborate the biosynthesis and accumulation of these compounds. The present study consists of a detailed analysis of the isoflavonoid pools in germinating lupin seeds as well as their root exudates, and emphasizes the putative role(s) these metabolites may play in the developmental processes of legumes, especially in relation to pathogen stresses and symbiosis.

The HPLC protocol developed for this study was successful in providing unambiguous identification and quantification of the constituent isoflavonoids in lupin tissues. The results obtained (Sections E.2.1. and E.5.) indicate a state of flux in synthesis, turn-over

and secretion, where the levels of individual groups of isoflavonoids as well as the total amounts in different parts of the seedling continued to change during growth and development. However, these variations appear to be time- and organ-specific, i.e. developmentally regulated. The total isoflavonoids identified were lowest in the dry seeds and soaked seedling parts, as compared with an active synthesis in the later stages of germination. The highest isoflavonoid content in the seedling parts analyzed occurred at day 2 in the root tip, and at day 3 in the other parts, whereas such maxima were observed at day 11 in the lateral roots. These maxima correlated with the appearance of chlorophyll in aerial tissues, and preceded primary and lateral root elongation. This suggests that isoflavonoid accumulation precedes both the photosynthetic activity and the increase in elongation in white lupin. Following the maximum levels observed in each part, there was a gradual decrease in total isoflavonoids (ca <2 nmol/mg f. wt.) in all seedling parts analyzed, except for the distal region of the primary root which remained high (ca >4 nmol/mg f. wt.). The exceptionally high isoflavonoid contents of the primary root distal, the site of lateral root formation (i.e. at days 6 to 8), further illustrates that isoflavonoid accumulation correlates with specific developmental events. In addition, pulse-labeling studies of intact seedlings with cinnamic acid demonstrated an active isoflavonoid biosynthesis from days 2 to 4, whose composition closely resembles that of the isoflavonoid content observed in non-labeled experiments. Furthermore, pulse labeling of lupin seedlings, followed by a chase in water, revealed that 10 day-old cotyledons no longer contained any label (data not shown). However, the variations observed and the turn-over of these metabolites in growing parts may be explained by their translocation to other parts of the seedling as well as their secretion into the rhizosphere.

Two trends can be observed which seem to correlate the amount of isoflavonoid groups with seedling growth and development. One, involves the predominance of glucosides (ca 75-95% of total), the lower levels of aglycones (ca 5-25% of total) and the traces of prenylated compounds observed during all growth stages (except for higher levels of

diprenyls, in some cases). The ratio of glucosides to aglycones observed varied extensively during growth of all tissue parts (Appendix P). This ratio was >4.4:1 in tissue parts where elongation takes place, whereas it was <4.4:1 in non-elongating parts such as the cotyledons, as well as the soaked parts, which did not yet start to grow. A similar trend of these ratios was also observed in the pulse-labeling of intact seedlings, where the ratio of glucosides to aglycones was 3:1 for cotyledons and 16:1 for roots. Very recently, Mo and co-workers showed that the addition of kaempferol to flavonoid-deficient (CHS-) petunia pollen grains rescued pollen function, mainly germ tube elongation (Mo *et al.*, 1992). This flavonol aglycone was shown to act as a signalling molecule in pollen tissue, whose effect resulted in both cell division and elongation of the germ tube. The 3-*O*-glucoside of kaempferol was not active as a signalling molecule, although it was 10-fold more abundant than its aglycone in wild-type petunia stigma and pollen (Mo *et al.*, 1992). Interestingly, the ratio of kaempferol 3-*O*-glucoside to kaempferol, reported in the latter study, is comparable to the isoflavone glucoside to aglycone ratio we observed in growing lupin tissues. These authors hypothesized that the inactive, glucosylated form of kaempferol may constitute a storage-pool, with ß-glucosidase being responsible for the production of the active aglycone. In alfalfa seed effusates, the ratio of the luteolin 7-*O*-glucoside to luteolin (a flavone) was found to be 40:1 (Hartwig *et al.*, 1991). Luteolin, but not its glucoside, is the inducer of *nod*-genes of *Rhizobium meliloti* (Peters *et al.*, 1985), and stimulates the growth of this bacterium in liquid culture (Hartwig and Phillips, 1991). In lupin, we have observed correlations between the appearance (or disappearance) of isoflavonoids and specific developmental stages. It may be envisaged that the aglycones may act as signalling molecules in developmental processes, or may be further substituted with prenyl groups to fulfill different functions. Taking into account the putative role of kaempferol in the development of petunia, we can speculate on similar effects of the isoflavonoids in lupin development. This model implies the existence of a developmentally-regulated ß-glucosidase activity responsible for the production of the

active aglycone(s), that is derived from a pre-existing glucoside pool. Following these lines, the observed ratios of glucosides to aglycones suggest that such an isoflavonoid-balance is required for normal development in lupin tissues.

The other trend can be observed for tissue parts where diprenylated compounds are more abundant. In dry seeds, the amount of diprenylated derivatives was found to be as abundant as the glucosides, and differs from that observed in the growing tissues. The relative abundance of diprenyls is reduced upon the soaking of seeds, which correlates with the high metabolic activity of the germination process and suggests that diprenylated compounds may act as a reservoir of pre-infectional compounds in lupin seeds, ready to be secreted. However, we can also speculate on the probable involvment of diprenylated derivatives in the maintenance of seed dormancy in lupin. Diprenylated compounds are also present at higher levels in the hypocotyls from days 1 to 3, but disappear with the onset of elongation, suggesting that their relative abundance may correlate negatively with hypocotyl elongation. Similar correlations can be observed with respect to the relative abundance of diprenyls and the elongation of the epicotyl, which emerges from the cotyledons only at day 7. The presence of diprenyls until day 7 in the primary root parts other than the tip, and their especially high levels at day 6-8 in the distal region, further suggest a correlation with the developmental processes leading to lateral root formation. Taken together, these results seem to indicate that a negative correlation exists between the high levels of diprenylated compounds in lupin tissues and the processes of seed germination, hypocotyl and epicotyl elongation, as well as their possible involvement in differentiation of lateral roots.

Considering the alterations observed in the amounts of diprenyls and the ratios of glucosides to aglycones, the question arises as to whether such compounds are involved in the regulation of growth and development of lupin. Plants produce a wide variety of secondary metabolites, many of which may belong to the same group of compounds, e.g. aglycones, glucosides, mono- and diprenyl derivatives of "isoflavonoids", notwithstanding

the different substituents of each of the latter. However, our lack of knowledge as to the exact function of each of these compounds makes it difficult, at the time, to assign a specific role for each metabolite. Furthermore, we cannot eliminate the possibility that the variations observed in the isoflavonoid constituents during development of lupin may be the consequence of growth processes.

The secretion of plant secondary metabolites, especially flavonoids, through the roots or leaf surfaces is a well known phenomenon by which plants exclude 'cytotoxic' metabolites away from the cytosol. The best known example is the secretion of flavonoid signal molecules into the rhizosphere in order to initiate and control the symbiotic processes (Mulligan and Long, 1985, Karr *et al.*, 1992). In addition, it is known that prenylation of flavonoids increases their lipophilicity and, therefore, their secretion, as well as increasing their potential role as phytoalexins (Ingham, 1983). The lupin system is no different and, in fact, lupin cell suspension cultures secrete the same pattern of isoflavonoids as those secreted by the intact, uninjured cultured-cells (Hallard *et al.*, 1991).

We have estimated a secretion rate of 0.23 nmol/seedling-hr in lupin seed effusates during the first hours following the onset of germination. Secretion by the root exudates remained lower than that value until day 7, probably due to the small size of the root system during this growth stage. However, the increase in secreted isoflavonoids observed by day 8 reflects the secretory capacity of a fully developed root system harboring both primary and lateral roots. The rates of flavonoid secretion reported in seed rinses (effusates) during the onset of imbibition for other Leguminosae varies from 0.87 nmol/seedling-hr in alfalfa (Hartwig and Phillips, 1991), to 491 nmol/seedling-hr in black-seeded common bean (Hungria *et al.*, 1991a). Considering the size of lupin seeds (which is comparable to that of common bean), and their low rates of secretion at the onset of germination (comparable to those of alfalfa), raises the question as to why lupin effusates contain such low amounts of isoflavonoids. It maybe envisaged that adaptation has favored a high isoflavonoid

synthesis in the early stages of germination (days 2 to 4), whereas the strong antimicrobial effect of low amounts of pre-infectional compounds in seeds can fulfill the requirement for phytoalexins in the seeds.

The relative abundance of glucosides in the root exudates remained low at all sampled stages, as compared with that of other compounds. However, it has been reported that such low levels of glucoside secretion may be due to ß-glucosidase activity in the seed rinses (Hartwig and Phillips, 1991), whose action may alter their flavonoid composition. Such activity has been demonstrated in alfalfa, where incubation of the ß-glucosidase containing seed effusates resulted in a decrease in luteolin 7-*O*-glucoside (Hartwig and Phillips, 1991). On the other hand, it has been shown that a higher amount of glucosides in the exudates results from 'leakage' of these metabolites due to damaged root cells under high stress conditions (Young *et al.*, 1982). Accordingly, secreted glucosidases may account for the very high level of aglycones observed in sterile lupin seed effusates at day 1, as compared with root exudates of the older seedlings which contained low relative amounts of aglycones. These exudates contained high levels of monoprenyls, with a striking predominance of those derived from genistein. However, it is not known whether the latter compounds are secreted as soon as they are synthesized, or if they are derived from a pre-existing pool. The unidentified, but abundant peak(s) which eluted at Rt=9 min on HPLC fractionation, may account for such a hypothetical isoflavonoid pool (i.e. which might be monoprenylated glucosylated derivatives).

The role of these isoflavonoids in both the effusates and the exudates of lupin seedlings may be envisaged to act as signal molecules in the establishment and control of symbiosis with *R. lupini*, or as pre-infectional compounds to protect lupin roots against pathogen infection. Further experiments will be required to determine the inducer molecule(s) of symbiosis in this system. This may be acheived by using an *R. lupini* strain, transformed with a plasmid construct of common *nod*-genes, and harboring a ß-galactosidase (*lacZ*)

reporter gene, such as that used with *R. meliloti* and alfalfa exudates (Mulligan and Long, 1985).

In contrast with other legumes, white lupin accumulates two groups of isoflavonoids that can be distinguished by the presence or absence of an hydroxyl group at position 2'. Therefore, the abundance of genistein derivatives relative to the total isoflavonoids identified may be used as an estimate of the overall 2'-hydroxylation level of the isoflavonoid pools analyzed. The relative abundance of genistein derivatives found in the different parts of lupin seedling varied significantly during growth. They constituted about 50% of the total isoflavonoids in both dry seeds and soaked cotyledons, whereas they reached 70% in other soaked parts analyzed. However, the levels of genistein derivatives increased to >85% of total isoflavonoids in the hypocotyls, epicotyls and root tissues. Pulse labeling of excised lupin roots also exhibited a higher incorporation of label into genistein derivatives (89%) as compared with that of excised cotyledons (52%). These results suggest that isoflavonoid synthesis favors the formation of genistein derivatives in growing tissues, as compared with non-growing, storage tissues of the cotyledon. In addition, the relative abundance of genistein derivatives observed in seed effusates and root exudates was similar to those observed in the soluble isoflavonoid pools. Therefore, a relationship seems to exist between the levels of 2'-hydroxy derivatives and the state of development of the seedlings. Whether the isoflavonoid 2'-hydroxylase enzyme is specific to the parent aglycone (genistein) and/or its prenylated derivatives is not yet known. However, the fact that the ratios of genistein- to 2'-hydroxygenistein derivatives change significantly during growth and development, both in the soluble fractions as well as the exudates, seems to indicate that this hydroxylation reaction plays a significant role in maintaining a balance between both groups of compounds.

As is the case for most other legumes (Long, 1989), *Lupinus albus* has the ability to establish a symbiotic interaction with *Rhizobium*. Variations in the isoflavonoid constituents of the symbiont-'stressed' as compared to the other stresses (i.e. $CuCl_2$, yeast extract or chitosan) of lupin seedlings may help distinguish between symbiotic phytoalexins and the 'classical' phytoalexins. Phytoalexins are widely reported as post-infectional compounds that are *de novo* synthesized, accumulated and secreted (Ebel, 1987; Ebel and Grisebach, 1988). Some of these compounds, isolated from lupin, have been shown to be fungitoxic (Harborne *et al.*, 1976).

We elicited lupin seedlings with $CuCl_2$, yeast extract, chitosan and *Rhizobium* in order to verify their responses to several stress factors by analyzing the accumulation and secretion of isoflavonoids in comparison with the pre-infectional levels. In addition, their effect on the growth rates of different bacteria was investigated in this work, especially with respect to the lupin symbiont. Elicitation would be expected to result in a higher total isoflavonoid content and a higher relative abundance of compounds which may act as phytoalexins. The analysis of root isoflavonoids from yeast extract-, chitosan- and $CuCl_2$-stressed seedlings, as well as their exudates, clearly indicates an elicitor activity in each case. The results of these treatments are almost similar in that they exhibit an increased isoflavonoid synthesis in root tissues. In addition, the predominant accumulation of monoprenylated compounds in stressed roots, especially those of 2'-hydroxygenistein, suggests a role in white lupin's response to stresses such as $CuCl_2$, yeast and chitosan. In fact, prenylated flavonoids fulfill the definition of phytoalexins, since luteone and licoisoflavone A (the 2'-hydroxygenistein monoprenyls) have been shown to be fungitoxic (Fukui *et al.*, 1973; Harborne *et al* 1976). Furthermore, we have demonstrated in this study the inhibitory effect of luteone on the growth rate of *R. lupini*, *R. meliloti* and *A. rhizogenes*. We have also shown that 2'-hydroxylupalbigenin (a diprenyl) significantly inhibits the *in vitro* growth of *B. subtilis*, *A. rhizogenes* and *R. lupini*. However, the seedlings stressed with $CuCl_2$, yeast and chitosan, as well as their exudates, did not exhibit

dramatic increases of diprenyls as compared with those of the monoprenyls. The labeled isoflavonoid composition of the roots resulting from pulse-chase in a $CuCl_2$ solution further supports the fact that stressed roots and their exudates contain higher proportions of monoprenyls than diprenyls. A recent report showed that $CuCl_2$ elicitation resulted in increases of 2'-hydroxygenistein monoprenyls in the roots of other white lupin cultivars (Shibuya *et al.*, 1992), although they did not report variations in the levels of diprenyls or glucosides. Our results are also in agreement with those obtained with the well characterized soybean stress response, where glyceollin biosynthesis was reported to increase upon elicitation with either *Phytophthora* spores or its cell wall fragments (Zähringer *et al.*, 1979). Glyceollin isomers are known to be fungitoxic (Zärhinger *et al.*, 1981) and bacteriostatic (Weinstein and Albersheim, 1983; Parniske *et al.*, 1991a).

Elicitor treatment also resulted in dramatic increases in the secretion of lupin isoflavonoids, with a predominant abundance of aglycones and monoprenyl derivatives. In some cases, however, glucosides were also abundant in the exudates of elicited seedlings. High levels of glucosides may have resulted from leakage by damaged root-cell membranes, thus affecting the normal composition of secreted isoflavonoids. This phenomenon was observed in the exudates of $CuCl_2$-treated lupin seedlings at high concentrations (i.e. 3.0 and 0.3 mM), as well as those of yeast extract elicited roots. Previous studies with chickpea cell cultures, indicated that 0.25% yeast extract exerted a very potent elicitor activity (Kessmann and Barz, 1987). Considering the high concentration of yeast extract (1%) used with lupin tissues, it would be reasonable to assume the release of glucosides in the exudates may have been the result of disrupted root cells leading to passive leakage of glucosides (Young *et al.*, 1982).

The alterations in flavonoid contents resulting from incubation of roots with their compatible *Rhizobium* have been documented in a number of cases such as *Lotus pedunculatus* (Cooper and Rao, 1992), *Glycine max* (Schmidt *et al.*, 1992) *Medicago*

sativa (Dakora *et al.*, 1993), and *Vicia sativa* (Recourt *et al.*, 1992). In this study, changes in the accumulation and secretion of isoflavonoids following incubation with *R. lupini* were observed. However, these changes differed markedly from those obtained with other elicitors described above (i.e. $CuCl_2$, yeast and chitosan), since the increases in total isoflavonoids were of much lower magnitude than those obtained with the other elicitors (i.e. $CuCl_2$, yeast or chitosan). Differences in secretion levels have also been reported for soybean, where the levels of glyceollin I remained several times lower after symbiotic infection than after pathogenic infection (Schmidt *et al.*, 1992). Our results, therefore, are comparable with those obtained for soybean, and demonstrate that early symbiotic infection in white lupin leads to lower accumulation and secretion of isoflavonoids as compared with elicitation by $CuCl_2$, yeast or chitosan.

Another difference from other stresses is the increase in the relative abundance of genistein monoprenyls in both the roots and their exudates when stressed with *R. lupini*. Interestingly, these compounds are the major constituents of exudates in the control uninoculated seedlings, which suggests that symbiotic stress does not alter qualitatively the isoflavonoid composition as compared with other stresses. These results are not unexpected, since *R. lupini* is a compatible lupin-symbiont so that infection is beneficial to the host, and thus should not lead to marked qualitative alterations. In fact, there was a 10-fold increase in the secretion of genistein monoprenyls after incubation with *Rhizobium*. These results are comparable to those of soybean, where the rate of glyceollin I secretion was found to be 50-fold higher when inoculated with *B. japonicum*, as compared with those of untreated seedlings (Schmidt *et al.*, 1992). The pulse labeling of isoflavonoids followed by a chase in a *R. lupini* suspension corroborates these results, since comparable increases in the secretion of labeled genistein monoprenyls were observed at day 10. The secretion of aglycones and diprenyls also increased in *Rhizobium*-stressed lupin, whereas no such increase was detected for the 2'-hydroxygenistein monoprenyls.

The comparison of symbiotic 'stress' with $CuCl_2$, yeast extract or chitosan stresses suggest a specificity in lupin with respect to the predominent isoflavonoid derivatives elicited. Symbiotic elicitation in lupin, results in increases of genistein monoprenyls and diprenyls, as compared with $CuCl_2$, yeast extract or chitosan, which resulted mostly in the elicitation of 2'-hydroxygenistein monoprenyls. In addition, the results show that the relative abundance of genistein derivatives in symbiont-stressed seedlings was maintained to levels similar to those of the controls, as opposed to those of other elicitors we used. Furthermore, these derivatives were maintained at higher levels in the exudates of *R. lupini* stressed-seedlings as compared with the controls. In contrast with lupin, it appears that elicitation with both symbiont and pathogens in soybean results in increased amounts of glyceollin I, which seems to modulate both processes with no apparent specificity. However, it has recently been demonstrated that different PAL and CHS genes are expressed in response to elicitation with symbiont or pathogens in soybean, suggesting that a specificity exists in this plant at the level of gene expression, but not at the metabolite level (Eastabrook and Sengupta-Gopolan, 1991).

Although no enzyme activities have been investigated, our result suggest that, in white lupin, isoflavonoid modifying enzymes such as the 2'-hydroxylase may modulate such specificity. Furthermore, the results obtained with symbiont-stressed seedlings contrast with those of other stresses, since they suggest a depletion of the existing isoflavonoid pool, as opposed to the *de novo* synthesis resulting from other stresses. In fact, the lower ratio of total incorporation into root isoflavonoids to those in the exudates observed in $CuCl_2$-stressed seedlings, as compared with other pulse-chase experiments, supports the above view. This suggests that $CuCl_2$-stress results in an increased biosynthesis (i. e. *de novo* synthesis) as opposed to secretion at the expenses of the pulse-labeled pools observed in other treatments (control and *R. lupini*). This is further supported by the decrease in total isoflavonoids of symbiont-stressed roots at day 10, which contrasts with that of other stresses.

In most cases reported for *Rhizobium*-legume interactions, the flavonoid aglycones were shown to be the inducers of the *nod*-genes, and that they *did* stimulate the growth of the symbiont *in vitro*. It may be tempting to speculate on the molecular nature of the inducer of *R. lupini* since genistein monoprenyls are secreted in abundance upon inoculation of the host with the symbiont. However, the effect of wighteone (a genistein monoprenyl) on the growth rate of *R. lupini* was found to be significantly inhibitory as opposed to stimulatory. Accordingly, genistein monoprenyls may rather act as symbiotic phytoalexins, and maybe involved in controlling the extent of infection. Lupalbigenin (a genistein-derived diprenyl) was found to be even more inhibitory to *R. lupini* growth than wighteone. These results suggest that a mild symbiotic phytoalexin may be preferentially used by lupin to control early *Rhizobium* infection.

Root nodules are symbiotic organs resulting from bacterial infection of legume root tissue, and their stages of maturation are reflected by their size-increase (Hirsch, 1992). They are found predominantly on lateral roots, or at the junction of primary and lateral roots (Libbenga and Bogers, 1974; Caétano-Anollès *et al.*, 1991). Lupin nodules display a different isoflavonoid composition, not only from that of lateral roots, but also from other tissues or exudates analyzed so far (except the dry seeds). In fact, the diprenyls and, to a lower extent, the monoprenyls are the most abundant isoflavonoids in nodules. 2'-Hydroxylupalbigenin constituted 30.5% of total isoflavonoids in older nodules, whereas that of lupalbigenin and monoprenyls decreased as the nodule size increased. Furthermore, lateral roots contained lower amounts of prenylated isoflavonoids, as compared to the nodules they harbor. However, the relative high abundance of aglycones in these lateral roots suggests that the latter compounds may act as a reservoir of metabolites to be prenylated and exported to the nodules. Nonetheless, these results indicate that the alterations in the isoflavonoid composition of the nodules and neighboring roots, are the consequence of bacterial infection which resulted in a higher relative abundance of

prenylated compounds. Furthermore, it is interesting to note that the variations in isoflavonoid composition among different nodule-sizes can be correlated with the different stages of their maturation. A similar situation has been reported in soybean, where glyceollin I accumulated in the nodules and its amount has been reported to increase as nodule function decreased (Karr *et al.*, 1992). This phytoalexin was also present in the neighboring lateral roots, but at a much lower level than in the nodules (Karr *et al.*, 1992). In addition, the amount of the aglycone precursor, daidzein, was found to rise before glyceollin I appeared (Karr *et al.*, 1992).

The fact that prenylated isoflavonoids of lupin significantly reduced the growth rate of *R. lupini* in liquid medium, suggests that these metabolites may be utilized by the host to control nodule-symbiont populations. Furthermore, as the nodules senesce, the most *in vitro* inhibitory of these compounds, 2'-hydroxylupalbigenin, increased in relative abundance at the expense of the other isoflavonoids. As compared with early infection by *R. lupini*, the low relative abundance of genistein derivatives in all nodule-size groups indicates a 2'-hydroxylation level similar to those observed in seeds and is comparable to that observed with other stresses (i. e. $CuCl_2$, yeast and chitosan). Such an increased level of 2'-hydroxy derivatives further illustrates the state of stress-impaired *L. albus* roots by symbiotic organs.

The particular isoflavonoid composition of lupin-nodule tissues may be explained by a phenomenon called "hypersensitivity", in which the infected root cell accumulates phytoalexins to phytotoxic levels (Collinge and Sluzarenko, 1986). This 'suicide' mechanism is believed to enable the plant to control the degree of infection. The very high levels of diprenylated compounds observed in nodules may exert a more toxic effect on the symbiotic organ than on the symbiont. The fact that the concentration of diprenyls required to inhibit the *in vitro* growth of the symbiont (i.e. 10 μM) is 150-fold lower than that found in nodule tissues supports this hypothesis. In addition, the lower ratio of diprenyls observed in younger nodules may promote the dedifferentiation of the cortical root cells

required for nodule formation (Dudley *et al.*, 1989; Sheres *et al.*, 1990), whereas a higher ratio correlates with their senescence. Therefore, these results indicate that isoflavonoids may play an impotant role in the control of symbiotic infection during the development, maturation and senescence of nodules.

G: CONCLUSIONS AND PERSPECTIVES FOR FUTURE WORK

The various analyses of lupin tissues reported in this work show that active isoflavonoid biosynthesis takes place during early stages of seed germination (from days 2 to 4), most of which accumulates as glucosides. This is followed by a general decrease in total isoflavonoids during later stages of germination, which may be ascribed to turn-over and/or secretion of these metabolites. Secretion of isoflavonoids under sterile condition has been demonstrated and mostly takes place during seedling growth in the form of genistein monoprenyls. Elicitation with $CuCl_2$, yeast and chitosan leads to an increased accumulation and secretion of monoprenylated derivatives, especially those derived from 2'-hydroxygenistein. In contrast, genistein monoprenyls are the most abundant compounds in symbiotic 'stresses' during early germination. The results suggest that *L. albus* responds with specific molecules to different environmental stresses (i. e. symbiotic or other elicitors used). Furthermore, the high relative abundance of diprenyls seems to correlate positively with the absence of growth in seeds, and negatively with cell elongation at early germination stages, with lateral root growth and nodule formation, and with senescence of old nodules; suggesting that diprenyls play a role in promoting such cellular processes. Premature senescence was also observed in some of the stressed seedlings (e.g. $CuCl_2$ and chitosan at day 10), and this correlated with high levels of prenylated compounds in these tissues.

This study has, therefore, enabled to depict a few putative roles for the groups of compounds analyzed, the glucosides may be considered as non-reactive pools (reservoirs) of isoflavonoids, but also as precursors of other isoflavonoids. The isoflavonoid-balance (between the glucosides and aglycones) seems to be required for normal growth of lupin tissues. The relative amounts of diprenyls seem to act as determinants of dormancy, absence of cell elongation, lateral root formation as well as nodule differentiation and senescence. This study also demonstrates the preferential secretion of monoprenylated

compounds; the role of genistein monoprenyls as mild symbiotic phytoalexins, as compared with 2'-hydroxygenistein monoprenyls which are more effective phytoalexins; and finally, the predominance of diprenyls in nodule tissues.

However, several questions have arisen from this work, and further experiments are required to investigate the various suggestions derived from these results. At the phytochemical level, this involves the preparative scale isolation and identification of the unknown peak (Rt=9) in order to assign a role for such compounds in lupin growth. At the physiological level, to investigate the effect of diprenyl compounds on the growth of young lupin parts, as well as to monitor the accumulation of diprenyls in maturing lupin pods and seeds. In addition, the blocking of isoflavonoid synthesis at the onset of germination may reveal whether an isoflavonoid pool is required for the normal development of lupin seedlings. At the biochemical level, the activities of both ß-glucosidase and 2'-hydroxylase(s) enzymes need to be characterized and monitored during growth and elicitation of seedlings, respectively, since the results obtained suggest their involvement in these processes. Finally, studies at the molecular level would involve the molecular cloning of pRmM57 in *R. lupini* in order to assay for induction (and inhibition) of *nod*-genes via ß-glalactosidase activity. Otherwise, investigations of the enzymology and molecular cloning of prenyltransferase(s) are being carried out by co-workers in Dr Ibrahim's laboratory.

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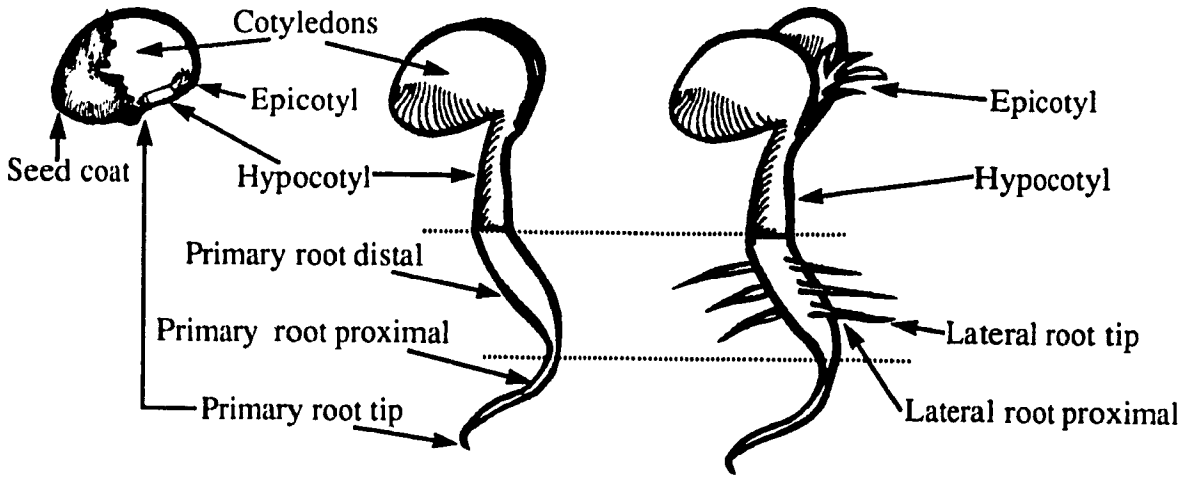
Appendix A: Number of determinations per seedling parts during growth

Seedling Parts	Stages of growth (days)															
	0	1	2	3	4	5	6	7	8	10	11	12	13	14	15	16	19
Sc[a]	1[b]	2	2	2	2												
Coty	1[b]	2	2	2	2			2		2							
Epi		2	2	2	2			2		2							
Hypo		2	2	2	4	3	1	1	1		1		1		1		1
Prd				2	4	3	3	3	3		2		2		2		2
Prp			2	2	4	3	3	3	3		2		2		2		2
Prt		2	2	2	4	3	3	3	3		2		2		2		2
Lrp											2		2		3		2
Lrt								3	3		2		2		3		2
Exu[c]	1	2	2	2	2	2	2	2	2	2		2	2	2	2	2	



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Appendix A: Continued

Values shown represent the number of isoflavonoid extracts analyzed by HPLC; each extract was made up of three seedling parts, according to Section D.3.1.

a- Abbreviations: Sc, seed coat; Coty, cotyledons; Epi, epicotyl; Hypo, hypocotyl; Prp, primary root proximal; Prd, primary root distal; Prt, primary root tip; Lrp, lateral root proximal;Lrt, Lateral root tip; Exu, exudates.

b- Dry seeds were extracted.

c- At growth stage 0, soaking water of 490 seeds was extracted at T=12 hr; at growth stage 1, soaking water of 100 seeds was extracted at T=24 hr; exudates from 38 seedlings were collected at all other growth stages.

Appendix B: Major isoflavonoid compounds in lupin tissues

Compound	Isoflavonoid group	Aglycone derivative
Genistein	Aglycone	----
2'-Hydroxygenistein	Aglycone	----
Lupinalbin A*	Aglycone	2'-Hydroxygenistein
Genistein-7-*O*-glucoside	Glucoside	Genistein
2'-Hydroxygenistein-7-*O*-glucoside	Glucoside	2'-Hydroxygenistein
Wighteone	Monoprenyl	Genistein
Isowighteone	Monoprenyl	Genistein
Lupiwighteone	Monoprenyl	Genistein
Licoisoflavone A	Monoprenyl	2'-Hydroxygenistein
Luteone	Monoprenyl	2'-Hydroxygenistein
Lupalbigenin	Diprenyl	Genistein
2'-Hydroxylupalbigenin	Diprenyl	2'-Hydroxygenistein

* Since lupinalbin A is a coumaronochromone, and not an isoflavone aglycone, it is not included in the aglycone group. However, values given for "total isoflavonoids identified" throughout the text include lupinalbin A.

Appendix C: Isoflavonoid content of dissected lupin seedlings and their exudates

Growth stage (days)	Seedling part name	7-O-glucosides		Aglycones			Monoprenyls		Diprenyls		TOTAL isoflavonoids identified	Genistein derivatives (%)
		Gen.	2'-OH-Gen.	Gen.	2'-OH-Gen.	Lupin-albin A	Gen.	2'-OH-Gen.	Gen.	2'-OH-Gen.		
0	Coty	0.02	0.07	0.00	0.02	0.00	0.00	0.00	0.07	0.01	0.20	46.6
1	Coty	0.01	0.01	0.01	0.02	0.00	0.00	0.00	0.01	0.00	0.06	49.1
2	Coty	0.10	0.02	0.03	0.03	0.00	0.00	0.00	0.00	0.00	0.18	73.7
3	Coty	1.01	0.94	0.30	0.37	0.07	0.00	0.01	0.00	0.00	2.71	48.7
4	Coty	1.21	0.80	0.35	0.31	0.07	0.01	0.01	0.00	0.00	2.74	56.9
7	Coty	0.81	0.25	0.20	0.05	0.01	0.01	0.03	0.00	0.00	1.35	75.4
10	Coty	0.80	0.41	0.22	0.13	0.00	0.00	0.01	0.00	0.00	1.59	64.6
0	Coat	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.03	36.2
1	Coat	0.04	0.01	0.02	0.01	0.08	0.00	0.00	0.06	0.00	0.23	53.1
2	Coat	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.05	63.0
3	Coat	0.08	0.01	0.04	0.01	0.01	0.00	0.00	0.01	0.00	0.16	82.6
4	Coat	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.07	51.7
1	Epic	0.12	0.10	0.04	0.06	0.00	0.02	0.01	0.24	0.00	0.58	71.6
2	Epic	2.57	0.25	0.80	0.08	0.00	0.00	0.01	0.15	0.00	3.86	91.4
3	Epic	10.08	0.26	0.67	0.05	0.01	0.01	0.00	0.25	0.00	11.33	97.2
4	Epic	2.97	0.24	0.40	0.03	0.01	0.02	0.00	0.25	0.00	3.94	92.6
7	Epic	3.07	0.35	0.47	0.03	0.03	0.02	0.03	0.00	0.00	4.01	89.0
10	Epic	4.26	0.36	0.45	0.02	0.06	0.01	0.01	0.00	0.01	5.19	91.1
1	Hypo	0.07	0.06	0.02	0.04	0.01	0.04	0.00	0.14	0.00	0.39	69.8
2	Hypo	8.70	0.48	0.48	0.07	0.00	0.01	0.01	0.17	0.00	9.92	94.4
3	Hypo	10.84	0.66	0.49	0.06	0.00	0.01	0.01	0.17	0.00	12.25	94.0
4	Hypo	5.26	0.43	0.38	0.03	0.01	0.02	0.01	0.06	0.00	6.20	92.3
5	Hypo	1.70	0.11	0.42	0.01	0.02	0.01	0.00	0.00	0.00	2.28	93.4

.../continued

Appendix C: Continued

Growth stage (days)	Seedling part name	7-O-glucosides		Aglycones			Monoprenyls		Diprenyls		TOTAL isoflavonoids identified	Genistein derivatives (%)
		Gen.	2'-OH-Gen.	Gen.	2'-OH-Gen.	Lupin-albin A	Gen.	2'-OH-Gen.	Gen.	2'-OH-Gen.		
6	Hypo	3.78	0.21	0.07	0.00	0.02	0.04	0.01	0.01	0.00	4.17	93.5
7	Hypo	3.79	0.32	0.11	0.01	0.03	0.00	0.00	0.00	0.01	4.27	91.3
8	Hypo	2.62	0.19	0.09	0.01	0.02	0.00	0.00	0.02	0.01	2.96	92.1
11	Hypo	3.01	0.44	0.05	0.01	0.02	0.00	0.01	0.01	0.00	3.56	86.4
13	Hypo	1.21	0.13	0.06	0.01	0.02	0.00	0.01	0.01	0.00	1.45	88.4
15	Hypo	1.47	0.34	0.03	0.02	0.00	0.00	0.01	0.03	0.00	1.91	80.4
19	Hypo	1.37	0.28	0.07	0.01	0.00	0.00	0.01	0.03	0.00	1.77	82.9
3	PRD	6.91	0.18	0.49	0.01	0.00	0.00	0.00	0.07	0.00	7.66	97.5
4	PRD	4.63	0.46	0.24	0.01	0.00	0.02	0.00	0.03	0.00	5.40	91.2
5	PRD	3.96	0.46	0.32	0.02	0.00	0.00	0.00	0.06	0.00	4.82	90.2
6	PRD	4.31	0.71	0.12	0.01	0.03	0.03	0.01	0.43	0.01	5.66	86.4
7	PRD	3.89	0.61	0.12	0.02	0.01	0.00	0.01	0.17	0.00	4.83	86.5
8	PRD	3.50	0.48	0.08	0.01	0.01	0.01	0.01	0.13	0.00	4.24	87.9
11	PRD	2.04	0.50	0.20	0.03	0.02	0.00	0.01	0.25	0.00	3.05	81.4
13	PRD	1.87	0.39	0.09	0.02	0.01	0.00	0.02	0.14	0.01	2.55	82.5
15	PRD	2.12	0.54	0.13	0.02	0.00	0.00	0.04	0.20	0.00	3.06	80.1
19	PRD	1.72	0.47	0.12	0.02	0.00	0.00	0.03	0.33	0.00	2.69	80.5
2	PRP	4.92	0.08	0.43	0.01	0.00	0.02	0.00	0.10	0.00	5.56	98.4
3	PRP	6.19	0.07	0.25	0.00	0.01	0.00	0.00	0.09	0.00	6.63	98.7
4	PRP	2.74	0.25	0.19	0.02	0.00	0.03	0.00	0.08	0.00	3.30	91.8
5	PRP	3.67	0.13	0.49	0.01	0.00	0.00	0.00	0.03	0.00	4.34	96.6
6	PRP	3.06	0.36	0.13	0.01	0.02	0.01	0.01	0.22	0.01	3.81	89.7
7	PRP	1.72	0.43	0.12	0.03	0.01	0.02	0.07	0.31	0.01	2.72	79.9

.../continued

Appendix C: Continued

Growth stage (days)	Seedling part name	7-O-glucosides		Aglycones			Monoprenyls		Diprenyls		TOTAL isoflavonoids identified	Genistein derivatives (%)
		Gen.	2'-OH-Gen.	Gen.	2'-OH-Gen.	Lupin-albin A	Gen.	2'-OH-Gen.	Gen.	2'-OH-Gen.		
8	PRP	1.28	0.28	0.03	0.02	0.07	0.02	0.06	0.23	0.01	2.00	77.9
11	PRP	1.16	0.23	0.14	0.03	0.03	0.09	0.04	0.71	0.02	2.43	86.1
13	PRP	1.66	0.27	0.03	0.02	0.00	0.04	0.03	0.12	0.01	2.18	84.6
15	PRP	1.37	0.31	0.02	0.02	0.01	0.03	0.04	0.08	0.00	1.89	79.9
19	PRP	0.57	0.30	0.12	0.02	0.00	0.01	0.04	0.64	0.01	1.71	78.7
1	PRT	0.32	0.14	0.08	0.04	0.02	0.01	0.00	0.00	0.00	0.61	67.8
2	PRT	4.71	0.17	0.50	0.00	0.00	0.06	0.00	0.37	0.00	5.81	97.0
3	PRT	2.61	0.13	0.53	0.00	0.00	0.00	0.00	0.01	0.00	3.28	96.1
4	PRT	1.55	0.17	0.25	0.00	0.00	0.00	0.00	0.01	0.00	1.97	91.3
5	PRT	2.21	0.44	0.06	0.00	0.00	0.00	0.00	0.00	0.01	2.71	83.5
6	PRT	1.62	0.24	0.01	0.00	0.00	0.00	0.00	0.00	0.01	1.89	86.7
7	PRT	1.12	0.22	0.02	0.00	0.00	0.04	0.00	0.00	0.01	1.41	83.8
8	PRT	1.58	0.28	0.03	0.00	0.00	0.00	0.00	0.02	0.01	1.91	85.0
11	PRT	1.04	0.09	0.03	0.00	0.00	0.03	0.00	0.00	0.01	1.19	91.4
13	PRT	1.19	0.35	0.01	0.00	0.00	0.21	0.03	0.03	0.01	1.84	78.7
15	PRT	1.22	0.19	0.03	0.01	0.01	0.06	0.00	0.32	0.58	2.42	67.1
19	PRT	0.40	0.20	0.03	0.01	0.02	1.32	0.05	0.44	0.02	2.50	88.0
7	LRT	1.33	0.20	0.13	0.00	0.00	0.00	0.01	0.10	0.00	1.78	88.0
8	LRT	1.23	0.35	0.12	0.00	0.01	0.00	0.01	0.09	0.00	1.82	79.3
11	LRT	1.54	0.29	0.13	0.01	0.01	0.12	0.03	0.39	0.01	2.53	86.0
13	LRT	1.41	0.61	0.07	0.01	0.01	0.04	0.05	0.12	0.00	2.32	70.8
15	LRT	0.91	0.26	0.03	0.01	0.01	0.06	0.07	0.21	0.00	1.56	77.5
19	LRT	0.85	0.36	0.16	0.03	0.01	0.08	0.08	1.75	0.04	3.36	84.6

.../continued

Appendix C: Continued

Growth stage (days)	Seedling part name	7-O-glucosides		Aglycones			Monoprenyls		Diprenyls		TOTAL isoflavonoids identified	Genistein derivatives (%)
		Gen.	2'-OH-Gen.	Gen.	2'-OH-Gen.	Lupin-albin A	Gen.	2'-OH-Gen.	Gen.	2'-OH-Gen.		
11	LRP	0.98	0.23	0.32	0.03	0.01	0.02	0.00	0.23	0.02	1.84	81.1
13	LRP	0.64	0.27	0.08	0.01	0.01	0.00	0.01	0.07	0.00	1.08	72.5
15	LRP	0.95	0.28	0.07	0.01	0.03	0.01	0.02	0.13	0.00	1.50	76.7
19	LRP	0.44	0.33	0.22	0.03	0.01	0.01	0.03	0.46	0.01	1.54	73.2
0.5	EXU	0.19	0.88	0.13	0.03	0.04	0.45	0.22	0.75	0.08	2.77	54.8
1	EXU	0.07	0.34	0.39	1.91	0.11	0.66	0.58	0.32	0.01	4.40	32.9
2	EXU	0.01	0.08	0.15	0.39	0.06	0.14	0.07	0.05	0.01	0.96	36.0
3	EXU	0.01	0.03	0.17	0.09	0.02	0.19	0.03	0.02	0.01	0.56	68.5
4	EXU	0.03	0.05	0.22	0.10	0.02	0.86	0.08	0.01	0.01	1.38	81.2
5	EXU	0.09	0.08	0.09	0.06	0.01	0.40	0.06	0.02	0.01	0.81	73.8
6	EXU	0.09	0.09	0.09	0.05	0.05	0.41	0.04	0.04	0.01	0.88	72.4
7	EXU	0.10	0.10	0.09	0.08	0.02	0.44	0.06	0.07	0.01	0.98	72.4
8	EXU	0.20	0.12	0.31	0.43	0.17	1.73	0.31	0.85	0.09	4.23	73.2
9	EXU	0.15	0.10	0.38	1.03	0.33	2.00	0.81	0.41	0.12	5.32	55.1
10	EXU	0.42	0.16	0.44	1.60	0.57	1.68	1.96	0.48	0.12	7.42	40.8
12	EXU	0.46	0.26	0.70	1.28	1.68	3.81	1.03	1.75	0.25	11.22	59.9
13	EXU	0.25	0.19	0.59	1.18	0.92	2.79	1.11	1.70	0.22	8.96	59.5
14	EXU	0.16	0.21	0.47	1.22	2.55	2.74	1.22	1.28	0.13	9.99	46.6
15	EXU	0.54	0.32	0.86	3.41	4.68	4.86	3.58	2.98	0.48	21.70	42.6
16	EXU	0.51	0.68	1.35	4.14	2.71	7.18	14.72	5.07	1.04	37.39	37.7

Values shown are in nmol/mg f. wt. for seedling parts and nmol/seedling for exudates. Seedlings were grown in vermiculite, whereas exudates were secured from seedlings incubated in water. Abbreviations: Gen., genistein; Coty, cotyledons; Coat, seed coat; Epic, epicotyl; Hypo, hypocotyls; PRD, primary root distal; PRP, primary root proximal, PRP, primary root tip; LRT, lateral root tip; LRP, lateral root proximal; Exu, exudates; see Appendix A.

Appendix D: Protocol of incorporation with a radiolabeled precursor[1]

Parameters	Treatments[2]				
	A	B	C	D	E
Sample size in seeds	1	1	4	4	4
Number of determinations	1	1	2	2	2
Parts analyzed by HPLC	2	2	2	2	2
Age at feeding (D[3])	2	2	2	2	2
Pulse feeding period (hr)	48	24	24	24	24
Chase feeding period (D)	-	-	7	7	7
Chase incubation medium[4]	-	-	H_2O	$CuCl_2$	*R. lup.*
Age at harvest (D)	4	3	10	10	10
Organ fed	root	segment	root	root	root
Amount fed (μCi)	1.0	0.625	0.25	0.25	0.25

[1]Tissues were incubated with [2-^{14}C] cinnamic acid. Superscript letters refer to standard deviations indicating confidence levels of: a, <5%; b, <10%; c, <15%; d, <20%; e, <25%; f, >25%.

[2]A and B are pulse labeling experiments; C, D and E are pulse-chase experiments.

[3]D, days.

[4]Sample C was chased in water; sample D in a solution of 30 μM $CuCl_2$; sample E in a *Rhizobium lupini* suspension.

Appendix E: Effect of of cinnamic acid and some flavonoids on bacterial growth rates

Flavonoid tested	Concentration in µM	Relative growth rates[1]				
		E. coli[2]	*B. subt.*	*A. rhizo.*	*R. meli.*	*R. lupini*
Cinnamic acid	0.1	0.86	0.68	1.01	1.01	0.99
	1.0	0.7	0.57	0.99	0.99	0.95
	10.0	0.73	0.54	1.13	0.97	0.76
Quercetin	0.1	0.78	0.86	0.97	0.97	0.91
	1.0	0.65	0.60	1.05	0.98	0.85
	10.0	0.72	0.59	1.11	0.94	0.74
Luteolin	0.1	0.89	0.79	1.00	1.02	0.92
	1.0	0.84	0.62	1.02	1.00	0.93
	10.0	0.79	0.57	1.19	0.98	0.64
Genistein	0.1	0.96	0.85	1.01	1.01	0.90
	1.0	0.89	0.71	1.06	1.01	0.83
	10.0	0.83	0.79	1.42	1.13	0.80
2'-Hydroxygenistein	0.1	0.90	0.87	0.96	1.01	1.03
	1.0	0.84	0.69	0.98	0.99	0.99
	10.0	0.92	0.64	1.32	1.00	0.79
Lupinalbin A	0.1	0.91	0.8	0.98	1.02	0.86
	1.0	0.85	0.68	1.05	1.01	0.89
	10.0	0.80	1.5	3.25	1.21	0.79

.../continued

Appendix E: Continued

Flavonoid tested	Concentration in µM	Relative growth rates				
		E. coli	*B. sub.*	*A. rhizo.*	*R. meli.*	*R. lupini*
Wighteone	0.1	0.83	0.73	1.04	1.02	0.85
	1.0	0.75	0.58	1.11	0.99	0.89
	10.0	0.73	21.8	1.48	1.17	1.40
Licoisoflavone A	0.1	0.87	0.87	0.98	0.92	0.77
	1.0	0.89	0.91	1.12	0.90	0.85
	10.0	0.90	2.19	1.24	0.95	1.39
Luteone	0.1	0.87	0.71	1.02	1.02	0.83
	1.0	0.81	0.63	1.03	1.00	0.99
	10.0	0.78	48.8	23.7	2.59	4.51
Derrone	0.1	0.87	0.73	1.08	1.0	1.05
	1.0	0.77	0.61	1.07	0.98	0.97
	10.0	0.76	981	1.15	0.96	0.86
Lupalbigenin	0.1	0.88	0.86	1.19	0.95	0.65
	1.0	0.89	1.0	1.09	0.93	0.77
	10.0	0.90	1.7	1.31	0.94	2.85
2'-Hydroxylupalbigenin	0.1	0.95	0.99	1.11	0.95	0.68
	1.0	0.93	1.29	1.28	0.96	0.77
	10.0	0.90	0.0	5.00	1.04	19.7

.../continued

Appendix E: Continued

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[1] Values shown are relative growth rates obtained by dividing the regression slope averages of triplicate growth curves (5 data points each) by the regression slope average of the relevant control. Therefore, controls have a value of 1.0 for every strain tested. Underlined values are significantly different from the control when processed with an ANOVA test (see Appendix F for confidence levels). The average growth rates with each MeOH control are: 1.87 ±0.02 for *E. coli*; 2.92 ±0.41 for *B. subtilis*; 1.85 ±0.03 for *A. rhizogenes*; 1.59 ±0.07 for *R. meliloti*; 4.58 ±0.31 for *R. lupini*. A volume of 30 µL of solvent was used for control and treatment assays. 50% DMSO was used for quercetin control and treatment assays, 80% MeOH for other compounds.

[2] Abbreviations: *E., Escherichia; B. subt., Bacillus subtilis; A. rhizo., Agrobacterium rhizogenes; R., Rhizobium; meli., meliloti.*

Appendix F: ANOVA tests comparing regression slopes of bacterial growth curves[1]

Escherichia coli

Assay groups and corresponding control

Quercetin group	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	3	1.294	0.431	19.64	.0005
Within groups	8	0.176	0.022	-	-

Other compounds group A[2]	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	24	1.171	0.049	21.59	.00005
Within groups	50	0.113	0.002	-	-

Other compounds group B[2]	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	9	0.258	0.029	3.53	.009
Within groups	20	0.163	0.008	-	-

Agrobacterium rhizogenes

Assay group and corresponding control

Quercetin group	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	3	0.118	0.039	29.73	.0001
Within groups	8	0.011	0.001	-	-

Other compounds group	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	33	8587626	260231	17.5	.00005
Within groups	68	1011210	14870	-	-

.../continued

Appendix F: Continued

Bacillus subtilis

Assay groups and corresponding control

Quercetin group	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	3	2.584	0.861	8.955	.0062
Within groups	8	0.77	0.096	-	-

Other compounds group A	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	24	5830745	242947	146.5	.00005
Within groups	50	82931	1658.6	-	-

Other compounds group B	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	9	72824411	8091601	1325413	.00005
Within groups	20	122.1	6.105	-	-

Rhizobium meliloti

Assay groups and corresponding control

Quercetin group	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	3	0.017	0.006	1.106	.4018
Within groups	8	0.042	0.005	-	-

Other compounds group	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	33	6.348	0.192	21.12	.00005
Within groups	68	0.619	0.009	-	-

.../continued

Appendix F: Continued

Rhizobium lupini

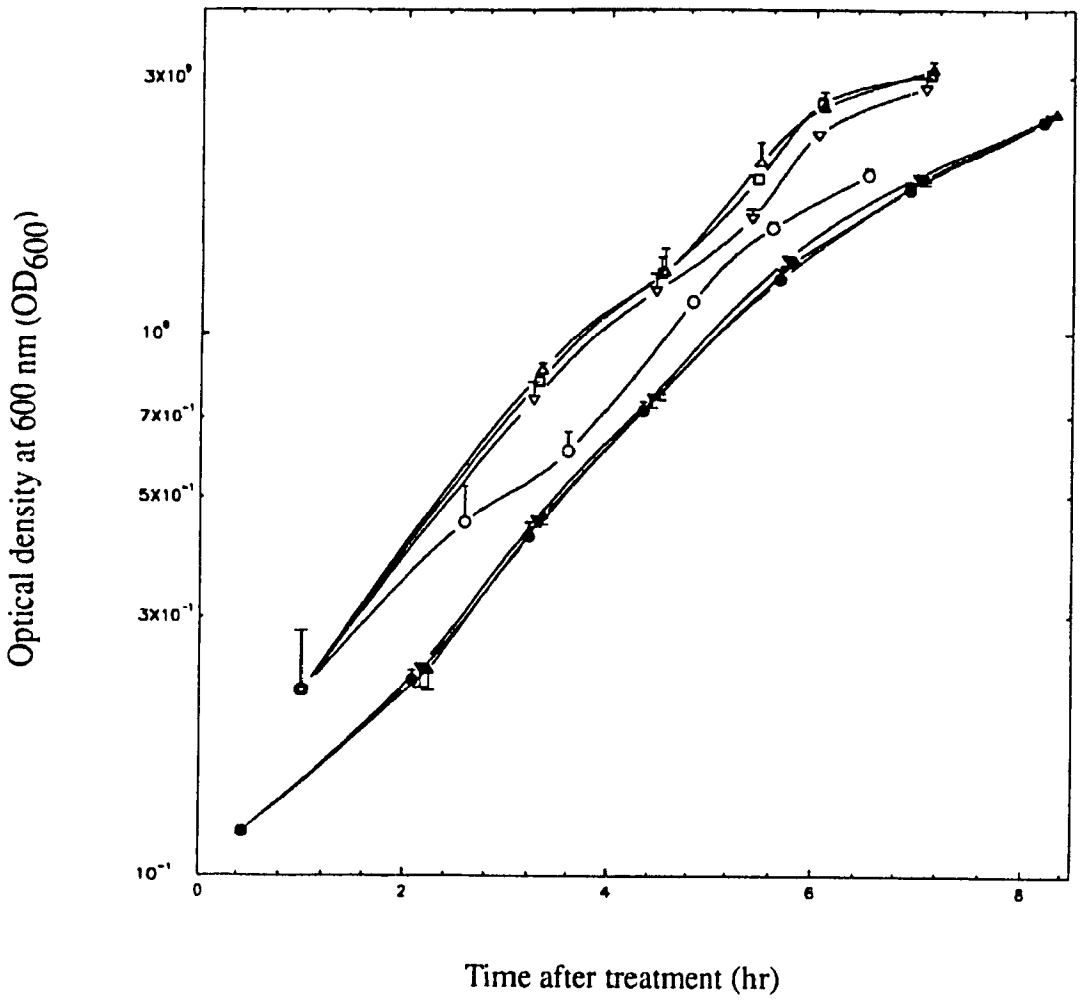
Assay groups and corresponding control

Quercetin group	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	3	93.56	31.19	82.31	.001
Within groups	8	3.04	0.38	-	-

Other compounds group	Degrees of freedom	Sum of squares	Mean squares	F ratio	F probability
Between groups	33	1357	41.14	97.3	.00005
Within groups	68	28.75	0.423	-	-

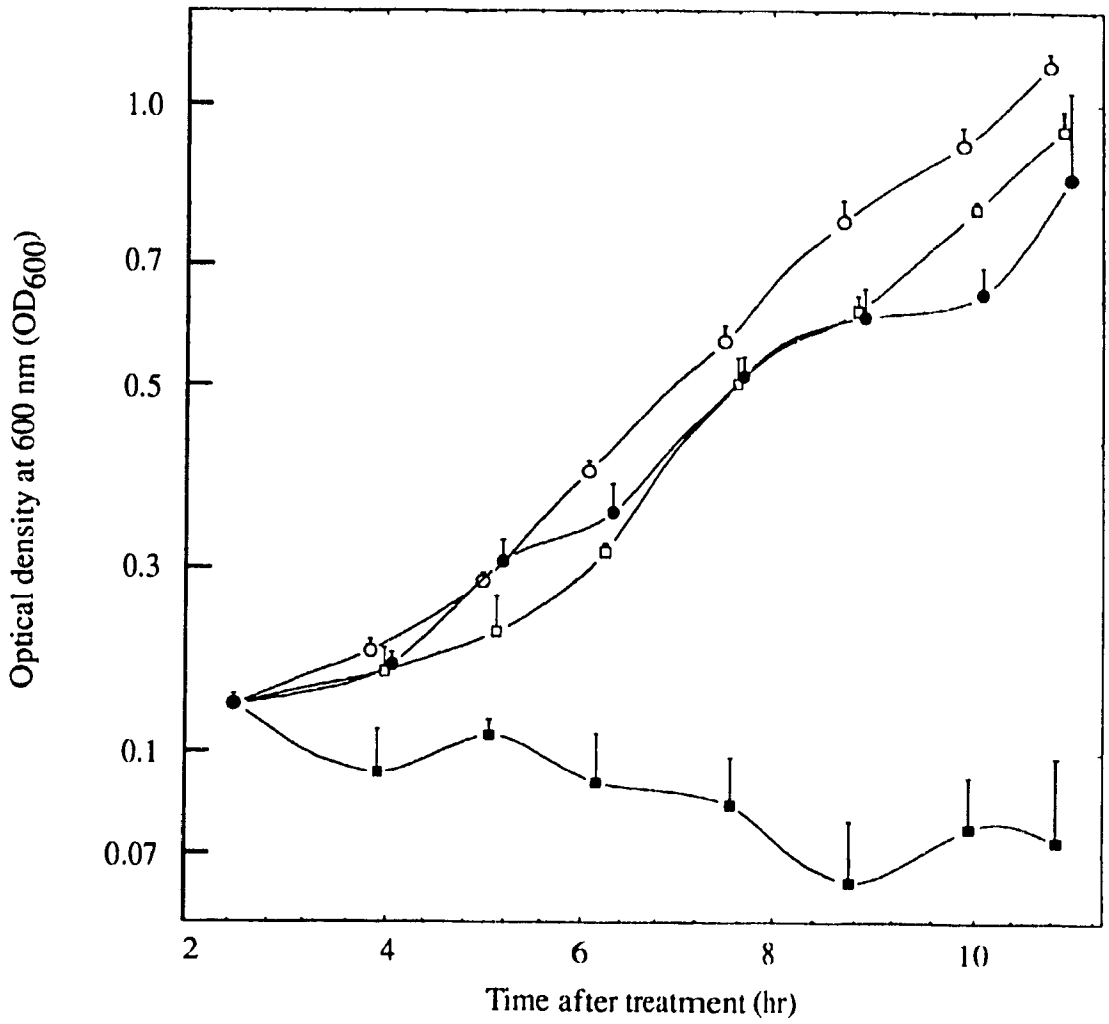
[1]The different parameters for all oneway analysis of variance (ANOVA) are presented for each set of control and treatments as triplicate regression slopes of bacterial growth curves, for each bacterial strain assayed.

[2]For both *E. coli* and *B. subtilis*, methanol soluble assays were not performed simultaneously, an extra control (e.g. triplicate curve) was therefore required. Group B includes licoisoflavone A, 2'-hydroxylupalbigenin and lupalbigenin; group A includes all other methanol soluble compounds assayed (except quercetin). For all other strains, all methanol soluble compounds were processed simultaneously.



Appendix G: Growth curves of *E. coli* in the presence of cinnamic acid, wighteone, lupalbigenin, luteone and 2'-hydroxylupabigenin at 10 μM in liquid medium

Legend:
(O) Control (for open symbols)
(●) Control (for solid symbols)
(□) Cinnamic acid
(△) Wighteone
(▲) Lupalbigenin
(▽) Luteone
(▼) 2'-Hydroxylupabigenin



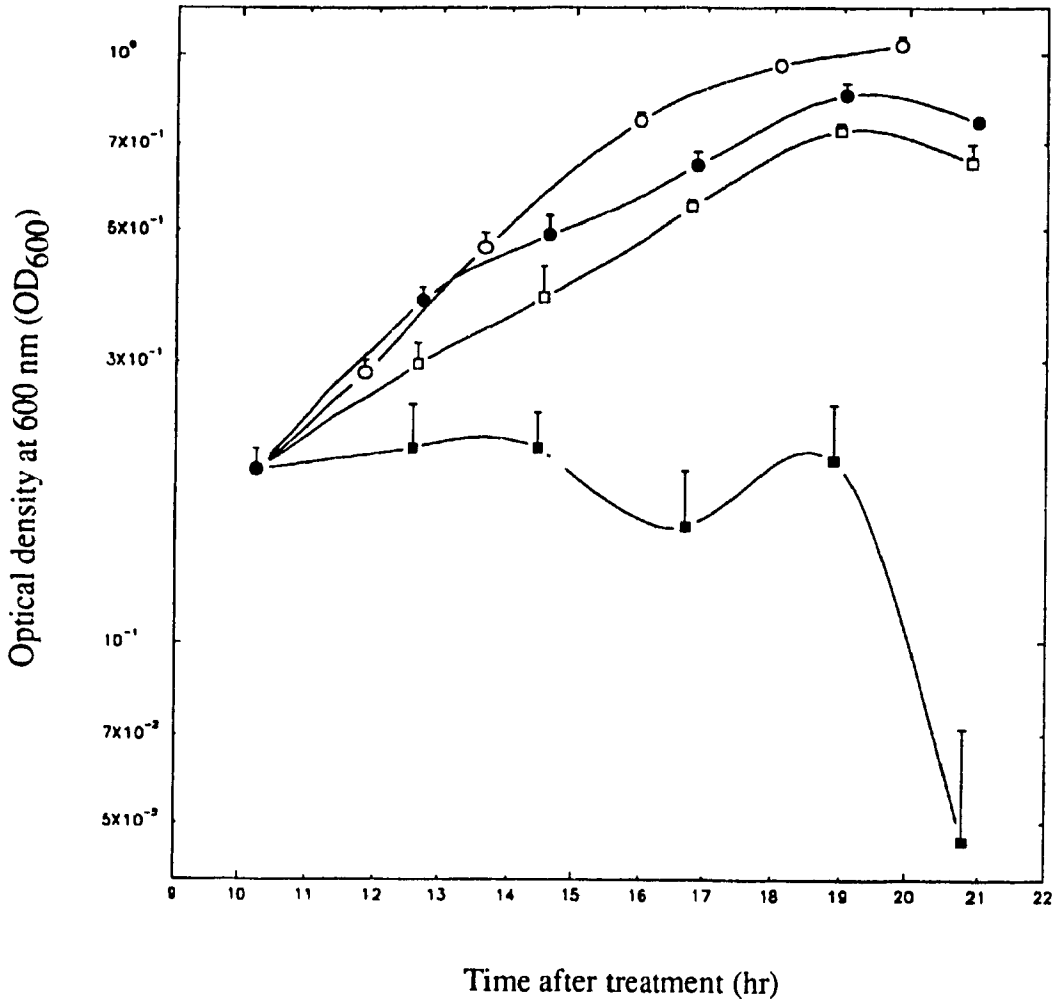
Appendix H: Growth curves of *B. subtilis* in the presence of licoisoflavone A, lupalbigenin and 2'-hydroxylupabigenin at 10 μM in liquid medium

Legend: (○) Control

(●) Licoisoflavone A

(□) Lupalbigenin

(■) 2'-Hydroxylupabigenin



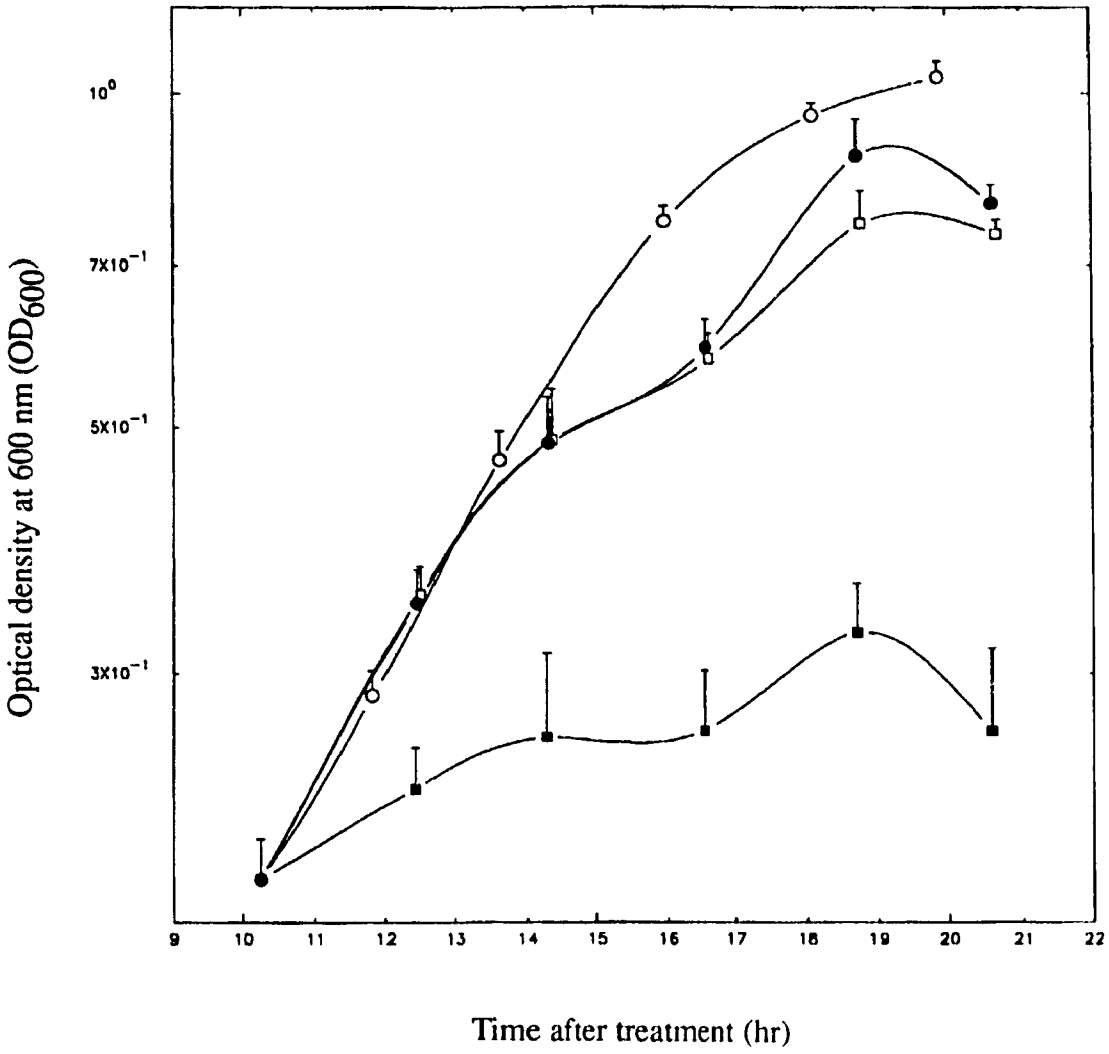
Appendix I: Growth curves of *A. rhizogenes* in the presence of licosisoflavone A lupalbigenin and 2'-hydroxylupabigenin at 10 µM in liquid medium

Legend: (O) Control

(●) Licoisoflavone A

(□) Lupalbigenin

(■) 2'-Hydroxylupabigenin



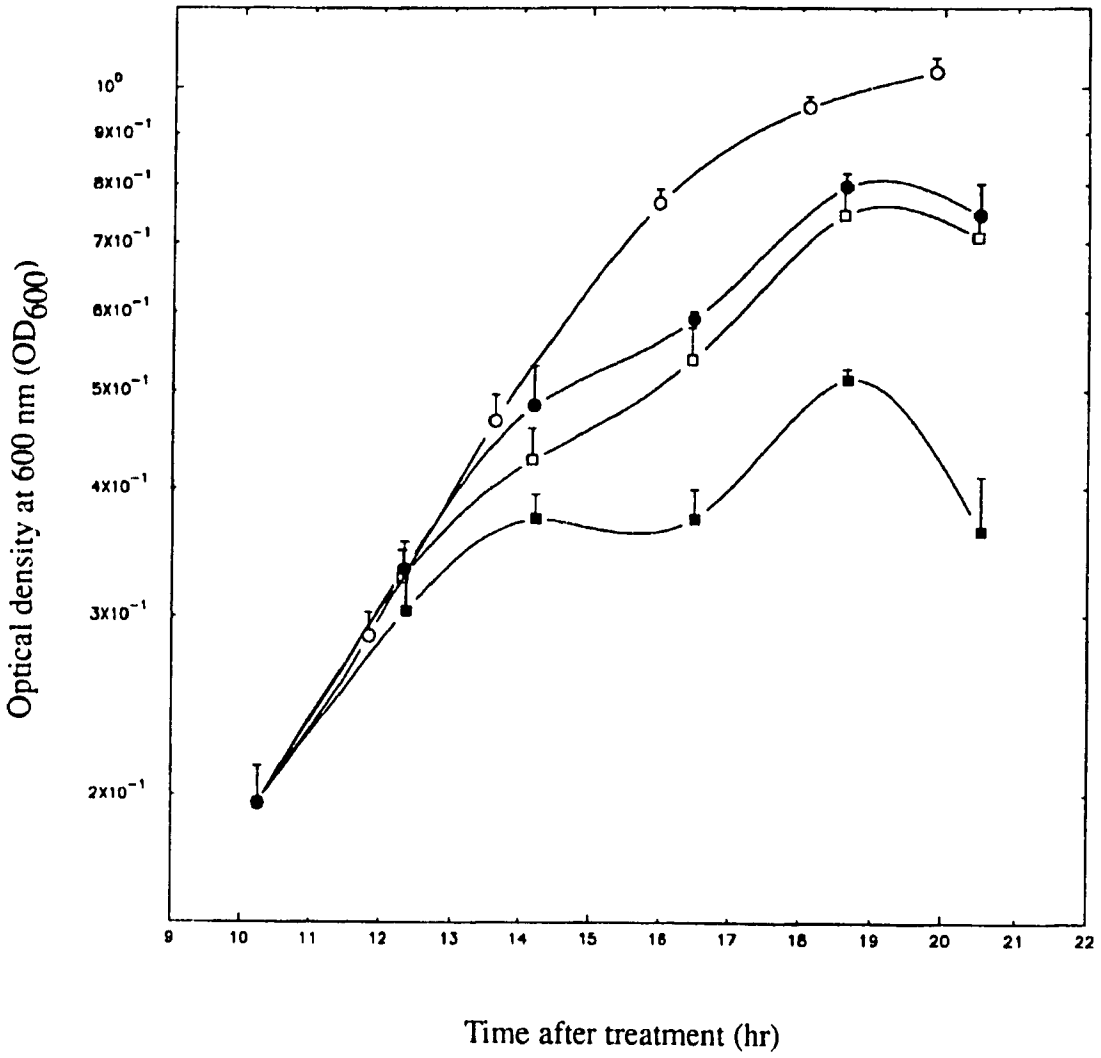
Appendix J: Growth curves of *A. rhizogenes* in the presence of derrone, wighteone and luteone at 10 μM in liquid medium

Legend: (O) Control

(●) Derrone

(□) Wighteone

(■) Luteone



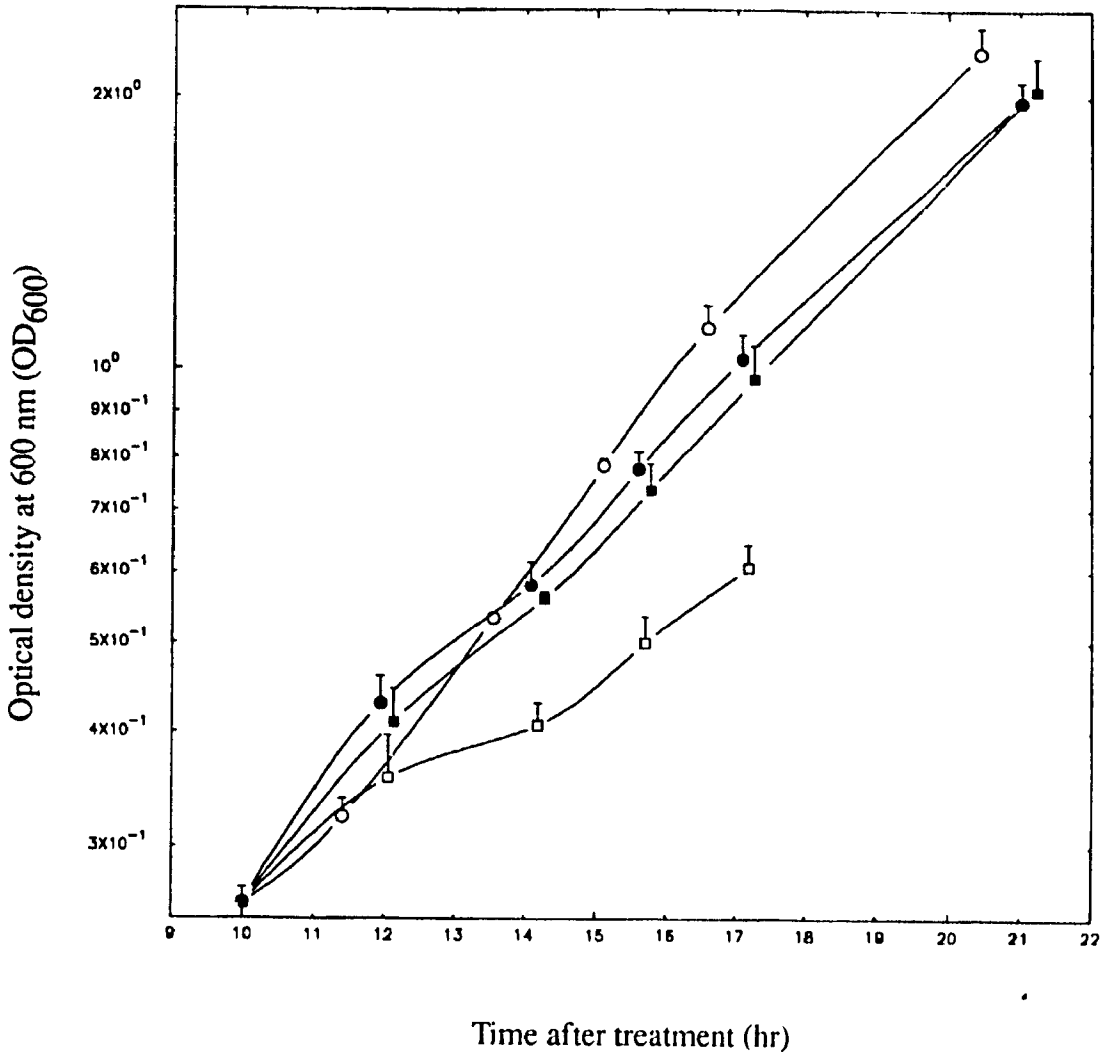
Appendix K: Growth curves of *A. rhizogenes* in the presence of 2'-hydroxygenistein, genistein and lupinalbin A at 10 µM in liquid medium

Legend: (O) Control

(●) 2'-Hydroxygenistein

(□) Genistein

(■) Lupinalbin A



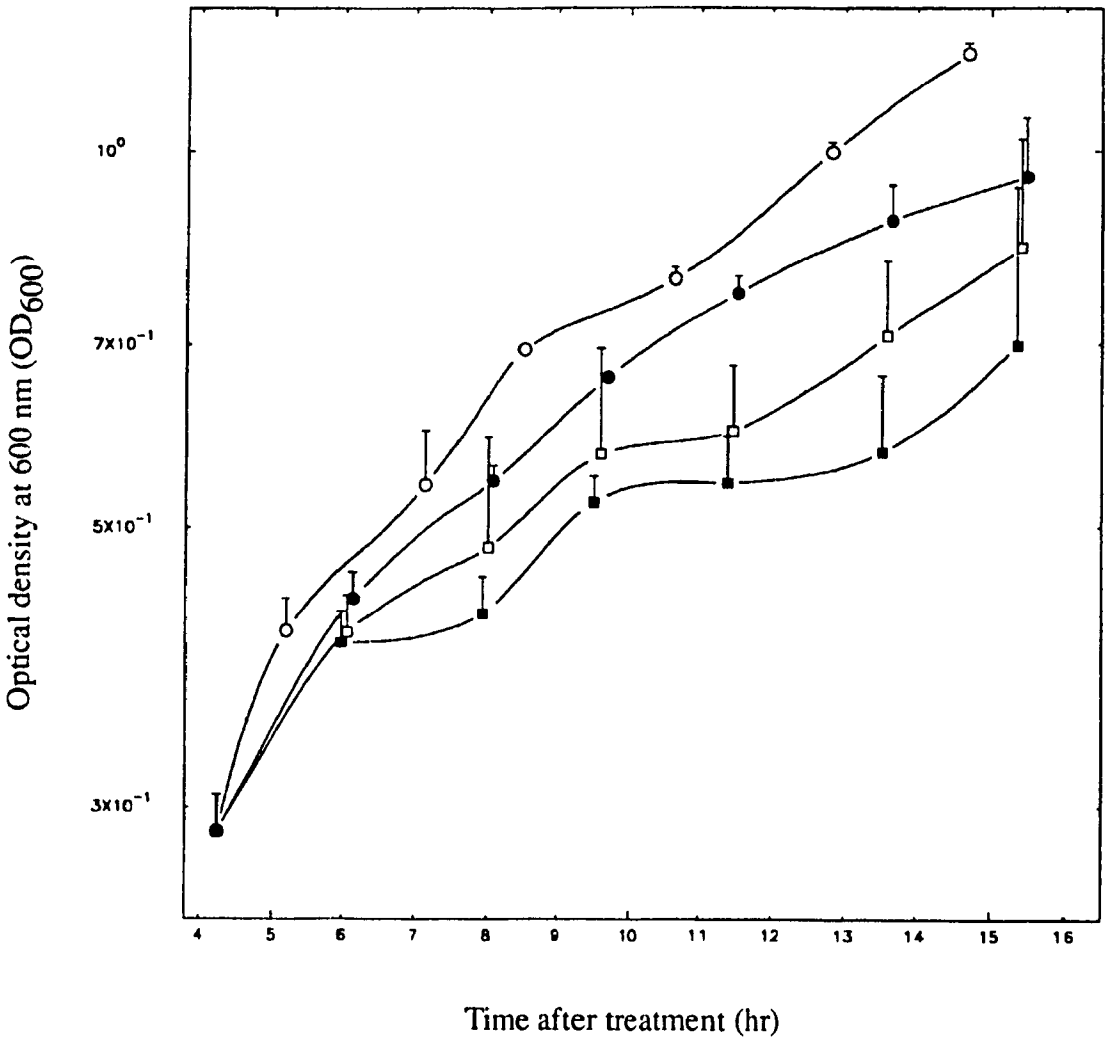
Appendix L: Growth curves of *R. meliloti* in the presence of genistein, luteone and wighteone at 10 µM in liquid medium

Legend: (O) Control

(●) Genistein

(□) Luteone

(■) Wighteone



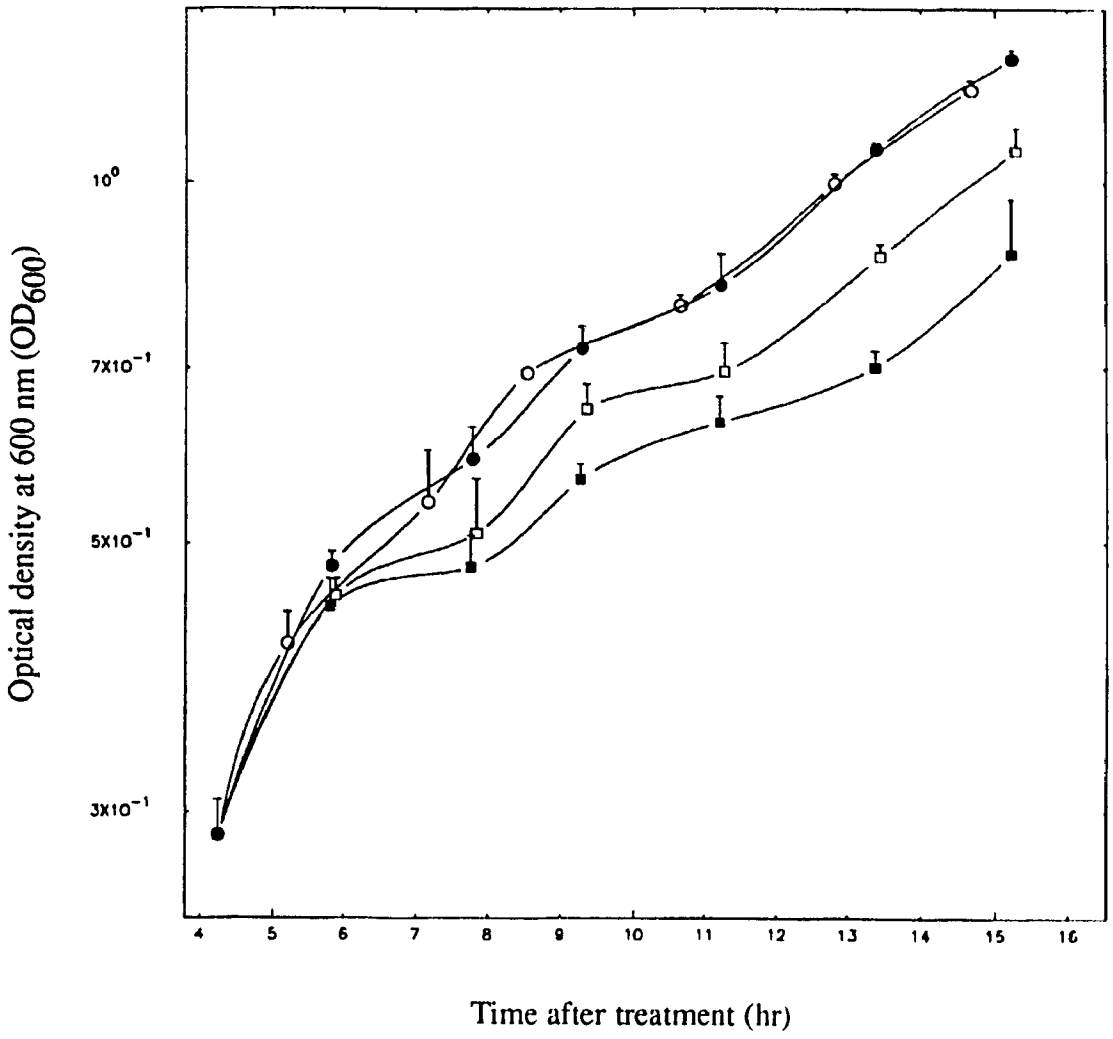
Appendix M: Growth curves of *R. lupini* in the presence of licosisoflavone A lupalbigenin and 2'-hydroxylupabigenin at 10 µM in liquid medium

Legend: (O) Control

(●) Licoisoflavone A

(□) Lupalbigenin

(■) 2'-Hydroxylupabigenin



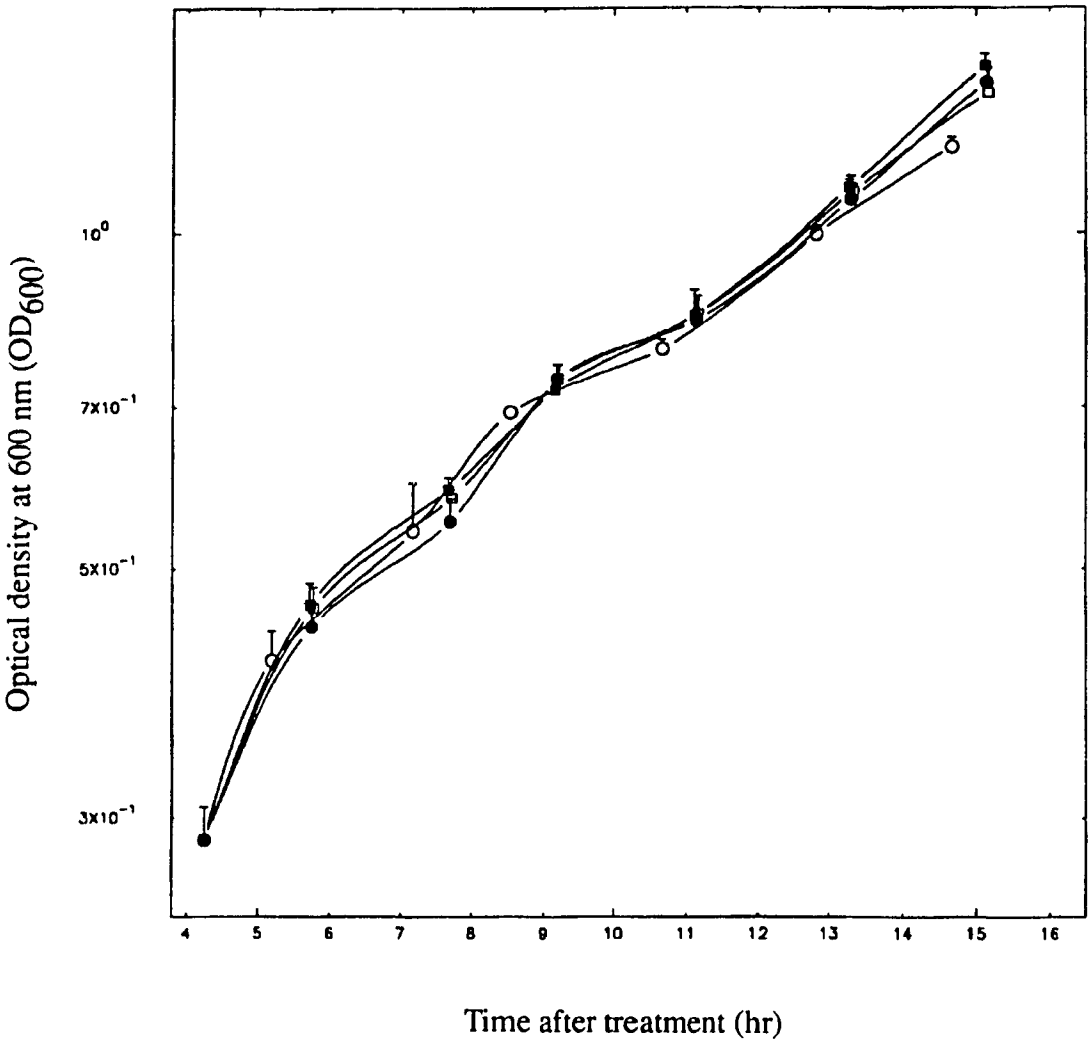
Appendix N: Growth curves of *R. lupini* in the presence of derrone, wighteone and luteone at 10 µM in liquid medium

Legend: (O) Control

(●) Derrone

(□) Wighteone

(■) Luteone



Appendix O: Growth curves of *R. lupini* in the presence of 2'-hydroxygenistein, genistein and lupinalbin A at 10 μM in liquid medium

Legend: (○) Control

(●) 2'-Hydroxygenistein

(□) Genistein

(■) Lupinalbin A

Appendix P: Ratio of glucosides to aglycones observed in seedling parts during growth

Seedling Parts	Stages of growth (days)															
	0	1	2	3	4	5	6	7	8	10	11	12	13	14	15	16	19
Coty	3.0	0.7	1.8	2.9	3.1			4.4		3.4							
Epi		2.5	3.1	14	7.4			7		9.7							
Hypo		2	16	21	14		52	33	28		52		18		38		20
Prd				14	20	13	38	32	44		11		21		18		16
Prp			11	24	14	7.6	26	15	28		8.4		39		39		6.3
Prt		4	10	5	7	46	>99	58	>99		42		>99		40		17
Lrp											3.6		10		12		3
Lrt								11	13		12		26		30		6

Values represent the ratio of the amounts of glucosides over that of aglycones. Shaded areas refer to parts and growth stages where this ratio is below 4.4, whereas the outlined areas refer to those with higher relative abundance of the diprenyls, see Discussion. Abbreviations: Coty, cotyledons; Epi, epicotyl; Hypo, hypocotyl; Prp, primary root proximal; Prd, primary root distal; Prt, primary root tip; Lrp, lateral root proximal; Lrt, lateral root tip.