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Clovers secrete specific phenolic compounds which either stimulate or repress *nod* gene expression in *Rhizobium trifolii*

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Rhizobium trifolii **mutants containing *Escherichia coli lac* gene fusions to specific nodulation (*nod*) genes were used to characterise phenolic compounds secreted from the roots of white clover (*Trifolium repens*) plants. These compounds either had stimulatory or inhibitory effects upon the induction of the *nod* genes. The stimulatory compounds were hydroxylated flavones and the most active compound was 7,4′-dihydroxyflavone. The inhibitory compounds present in white clover root exudates were umbelliferone (a coumarin) and formononetin (an isoflavone). Transcriptional activation of *nod* gene promoters in response to short exposures (3 h) of 7,4′-dihydroxyflavone was growth phase dependent; cells in early log phase were highly responsive to flavone additions *in vitro* and *nod* gene induction could be detected within 20 min of exposure at 5×10^{-7} M. Cells in other growth phases were generally unresponsive. A 10-fold molar excess of umbelliferone to 7,4′-dihydroxyflavone resulted in complete inhibition of *nod* gene induction. Some commercially-obtained flavones were found to have weak stimulatory activity but could also inhibit *nod* gene induction by more effective stimulatory compounds. Strong stimulatory and inhibitory compounds all possessed a 7-hydroxy moiety and showed other structural similarities. This suggested that there was one binding site for these compounds. Because the response to these compounds was rapid, we propose that these phenolics act at the bacterial membrane or that an active uptake system is involved.**

Key words: plant phenolics/*lac* fusions/flavonoids/coumarins

Introduction

The formation of nitrogen-fixing root nodules on legume plants occurs in response to infection by specific bacteria of the genus *Rhizobium* (Vincent, 1980). *Rhizobium trifolii* infects clovers, but not closely related legumes such as peas or alfalfa (Vincent, 1980). Recently it has been shown that an exchange of signals occurs between the interacting organisms. The plant releases flavones (low mol. wt plant phenolics) from its roots which stimulate the transcription of bacterial nodulation genes on the symbiotic plasmid (Redmond *et al.*, 1986; Peters *et al.*, 1986; Firmin *et al.*, 1986). Homologous (compatible) *Rhizobium* strains cause root hair distortion and penetrate root hair cells within 24 h (Turgeon and Bauer, 1982). Most incompatible *Rhizobium* strains are incapable of initiating these early infection events (Yao and Vincent, 1969; Djordjevic *et al.*, 1986) despite several *nod* genes being functionally interchangeable between many *Rhizobium* species (Banfalvi *et al.*, 1981; Kondorosi *et al.*, 1984; Djordjevic *et al.*, 1985a, 1986; Wijffelman *et al.*, 1985).

At least eight, and possibly >10, plasmid-borne genes are involved in nodule induction on specific plants (Rossen *et al.*, 1984; Török *et al.*, 1984, Schofield and Watson, 1986; Evans and Downie, 1986; Shearman *et al.*, 1986). In *R. trifolii*, six genes, called *nodABCDEF*, have been defined by DNA sequencing and mutational analysis. They are organised into three operons, *nodABC, nodD* and *nodFE* (Djordjevic *et al.*, 1985b; Innes *et al.*, 1985; Schofield and Watson, 1986). In addition nodulation genes are found in *R. trifolii* on the symbiotic plasmid in regions currently designated as II, IV and V (Djordjevic *et al.*, 1986). The *nodFE* (region III genes) and genes in regions IV and V confer host specificity (Djordjevic *et al.*, 1985b, 1986) while region II genes appear to be functionally homologous to the *R. leguminosarum nodIJ* genes (Evans and Downie, 1986).

Several reports have shown that only *nodD* is expressed in the absence of plant signal compounds, and that both the *nodD* gene product and plant-secreted flavones are required for induction of the other *nod* genes (Innes *et al.*, 1985; Rossen *et al.*, 1985; Mulligan and Long 1985; Redmond *et al.*, 1986; Peters *et al.*, 1986; Shearman *et al.*, 1986; Firmin *et al.*, 1986). Recently, the plant signal compounds have been identified as 7,4′-dihydroxyflavone, geraldone and 4′-hydroxy-7-methoxyflavone in white clover (Redmond *et al.*, 1986), luteolin in alfalfa (Peters *et al.*, 1986), and Firmin *et al.* (1986) have reported that several flavones as well as apigenin-7-O-glycoside stimulated *nod* gene expression in *R. leguminosarum.* These compounds are synthesised from the same precursors as the phytoalexins (Vickery and Vickery, 1981). The plant-secreted phenolic compounds which stimulate virulence gene expression in *Agrobacterium* (Stachel *et al.*, 1985) have chemical structures that are distinctly different from the flavones.

In this paper we show that expression of the *R. trifolii nod* genes is affected by the secretion of both stimulatory and inhibitory compounds from the plant and is also a function of the growth stage of the bacteria. Over 60 commercially-available compounds have been used to examine the effect of changes in chemical structure on the stimulatory activity of flavones and related compounds. Several compounds were found to antagonise the stimulatory activity of 7,4′-dihydroxyflavone; two of them, umbelliferone and formononetin, were present in root extracts from white clover. Umbelliferone proved to be highly antagonistic of *nod* gene induction by flavones *in vitro* and we proposed that it is responsible for the inhibition of bacterial *nod* gene expression which occurs in the vicinity of the root tips of clovers (Redmond *et al.*, 1986).

Results

Dependence of nod gene expression on growth phase

The expression in *R. trifolii* of the *nodD* gene was monitored at all stages of growth both in the presence and absence of flavone. The amount of activity of β-galactosidase (*nodD* expression) was maximal in cells in early log phase (Figure 1) but re-

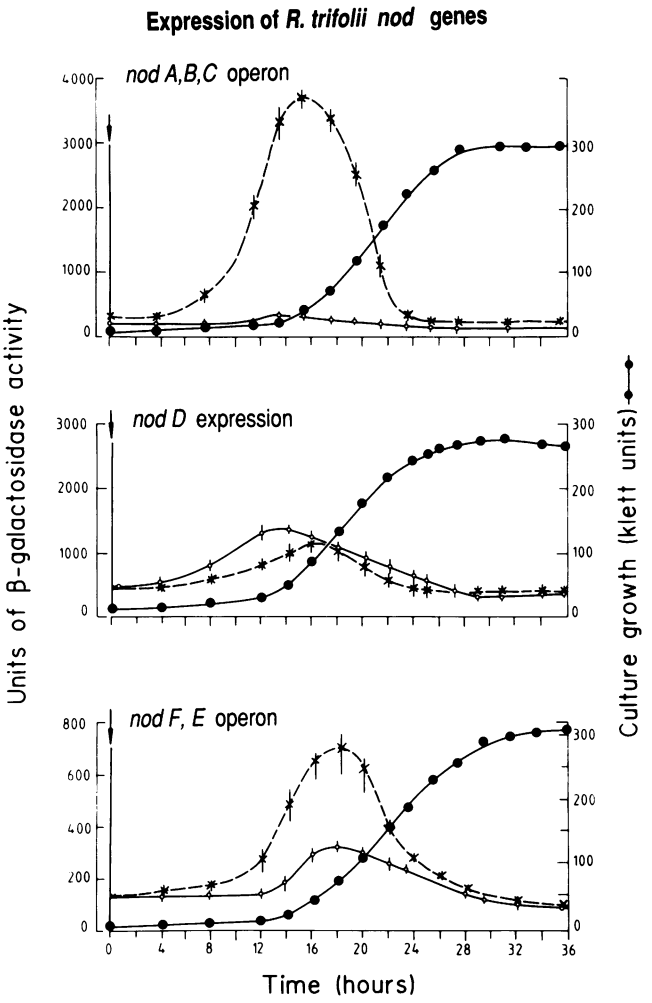

Fig. 1. Expression and induction of *R. trifolii nod* genes during the growth cycle in the presence and absence of flavones. The expression of the *nodD* gene is constitutive (at 400–500 units of β-galactosidase activity) in the presence (x) and absence (o) of flavone, except that the amount of *nodD* product per cell increases 2.5-fold for cells in early log phase. The inducibility of *R. trifolii* cells containing a *nodA::lac* fusion (*nod*218) or a fusion to the *nodFE* operon (*nod*1027) is also indicated in the presence (x) and absence (o) of flavone. Cells taken at various times during the growth cycle (measured in Klett units –●–) were exposed (x) or not exposed (o) to 5×10^{-7} M 7,4'-dihydroxyflavone for 3 h before the level of *nod* gene induction was measured. Only early log phase cells respond to the presence of the flavone. Note that different scales have been used for the *nodA* and *nodF* results.

mained constant at about 400–500 units for other growth phases. This result was consistent with other observations which indicate that there is a constitutive level of *nodD* expression (Innes *et al.*, 1985).

Since the root hairs of many legume species are transiently infectable for periods as short as 6 h and the addition of wild-type rhizobia to plants results in rapidly induced plant responses (Bhuvaneswari *et al.*, 1980; Ridge and Rolfe, 1985), we exposed culture-grown cells for short time periods to 7,4'-dihydroxyflavone to determine if *Rhizobium nod* gene expression could be rapidly induced. The ability of growing cells to respond to the presence of flavone over a 3 h period was determined using strains with either a *nodA::lac* fusion or a fusion to the *nodFE* operon. The inducibility of the promoters of the *nodA* and *nodF* genes was not constant. Cells in early log phase were particularly responsive to the exposure to the flavone while

Table I. Maximum induction levels achieved with various flavonoids

Compound	Maximum level of activity[a]	Concentration giving maximum activity (M)	Activity at 5×10^{-7} M
7-OH favone	6800	5×10^{-6}	3800
7,4'diOH flavone	15 000	1×10^{-6}	7000
7,4-'diOH flavanone	7100	5×10^{-5}	5500
geraldone	5800	3×10^{-6}	4200
chrysin	1600	5×10^{-5}	1200
apigenin	8500	5×10^{-6}	6000
luteolin	3000	5×10^{-6}	700
naringenin	3800	5×10^{-6}	1200
kaempferol	600	5×10^{-6}	400
quercetin	500	5×10^{-4}	140
geraldone chalcone	370	5×10^{-5}	290
taxifolin	400	5×10^{-5}	N.D.

[a]β-galactosidase activity was measured according to Miller (1972). All activities recorded after 4 h exposure of compounds to 400 μl of early log phase cells. The *nodA218* transcriptional fusion derivative was used to generate the results. Note that unsubstituted flavone (not listed) has no inducing activity.
N.D. = not determined.

cells in other growth phases showed a markedly reduced response or did not respond at all (Figure 1). A substantial increase in the induction of the *nod* gene promoters (15-fold for *nodA* and 2.5-fold for *nodF*) occurred when 7,4'-dihydroxyflavone was added to early log phase cells at a concentration of 5×10^{-7} M (Figure 1). The stage of growth which showed enhanced induction of *nodA* and *nodF* genes corresponded to the period where the maximal amount of *nodD* expression occurred. Note that a small, but reproducible, amount of β-galactosidase activity was also detected in the absence of flavone in early log phase cells for the strains carrying *nodA* and *nodFE:: lac* operon fusions.

Gene induction by substituted flavones and related compounds

Despite their structural similarity, the three flavones isolated from white clover root extracts, 7,4'-dihydroxyflavone, geraldone (7,4'-dihydroxy-3'methoxyflavone) and 4'-hydroxy-7-methoxyflavone, differ in their ability to induce transcription of the *nod* genes in *R. trifolii* (Redmond *et al.*, 1986). In an attempt to define the structural properties responsible for the variation of activity, several commercially available compounds were tested for their ability to induce *nod* gene expression. The activity of β-galactosidase produced by cells containing a *nodA::lac* operon fusion was measured for a range of concentrations of the test compound and these results are presented in Table I.

The compounds which produced a significant enhancement of *nod* gene expression were all flavonoids with a hydroxyl group at the 7 position (Figure 2). Isoflavones (biochanin A, diadzein), coumarins (umbelliferone and coumarin), coumeric acids (*ortho-*, *meta-* and *para*-coumeric acids), and hydroxybenzoic acids, (2,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 3'4'-dihydroxybenzoic acid and *p*-hydroxybenzoic acid), had little or no stimulatory effect. Coumeric acids are key metabolites in the synthesis of flavones and isoflavones while the benzoic acid derivatives are common breakdown products of flavones (Vickery and Vickery, 1981).

Two 7-hydroxyflavonoids, morin and myricetin, did not induce *nod* gene expression. There are several possible explanations for this lack of activity, including limited solubility. For this reason it is safer to draw conclusions from the properties of these molecules that do stimulate gene expression. Nevertheless, chang-

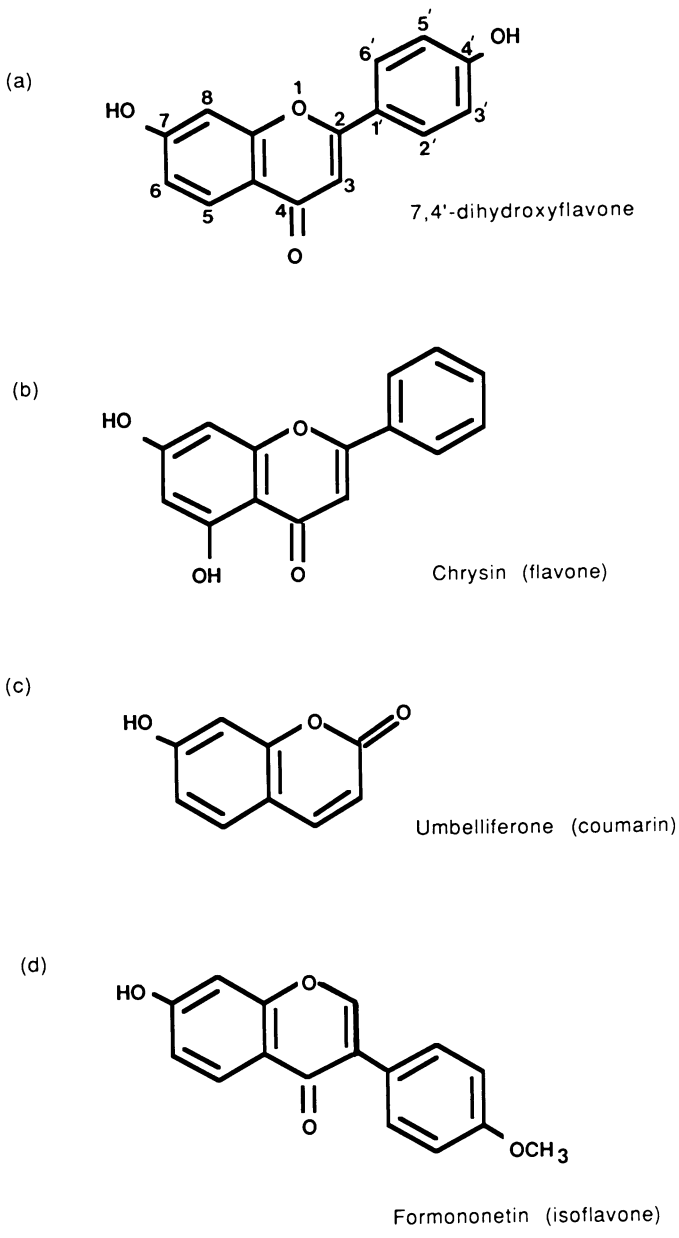

Fig. 2. Structures of stimulatory and inhibitory plant phenolic compounds. (a) Structure of the clover-derived flavone, 7,4′-dihydroxyflavone showing the numbering system used to define ring positions.

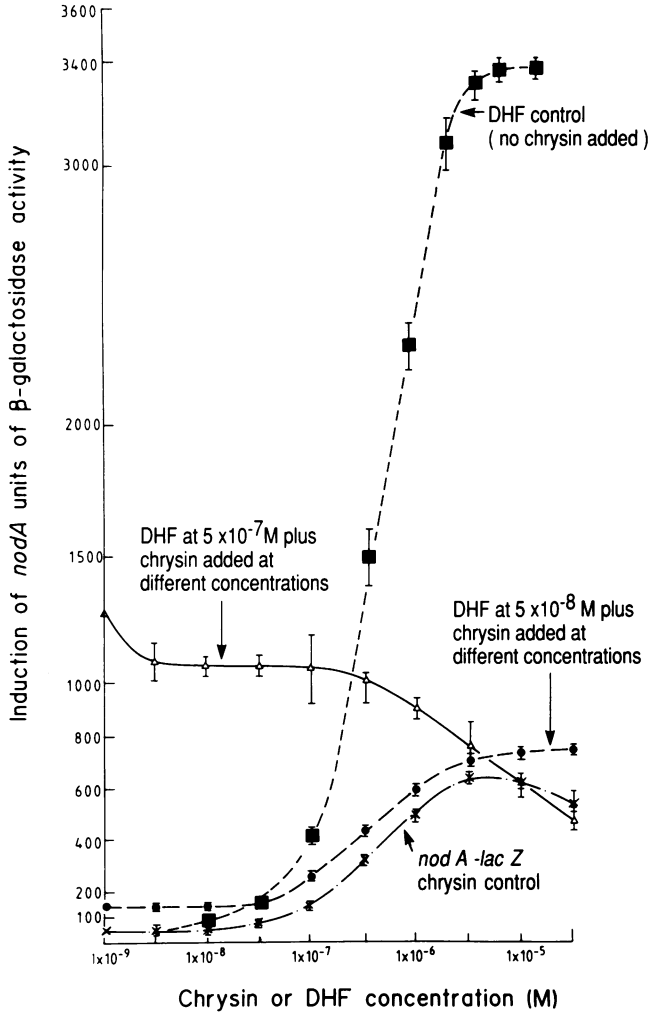

Fig. 3. The effect of chrysin addition on *nodA* induction mediated by 7,4′-dihydroxyflavone. The dose/response of the translation fusion derivative *nodA114* to either chrysin (x) or 7,4′-dihydroxyflavone (■) is shown. When added alone, chrysin and 7,4′-dihydroxyflavone both begin to induce *nodA* expression at the same concentration (~5 × 10^{-8} M) but chrysin does not induce the *nodA* gene to the same extent. When the concentration of 7,4′-dihydroxyflavone is held constant at 5 × 10^{-7} M (△) or 5 × 10^{-8} M (•), and cells are exposed to increasing amounts of chrysin, the level of induction approaches that achieved by the addition of chrysin alone. Thus chrysin has stimulatory activity but can also inhibit 7,4′-dihydroxyflavone-mediated induction. At least three replicates were performed to generate the results in this figure. Repeat experiments generated similar responses.

ing the 7 substituent from hydroxy to methoxy (4′-hydroxy-7-methoxyflavone or tectochrysin) or to O-glycosyl (naringin, diosmin, apiin or rhiofolin) results in a substantial reduction in, or elimination of, stimulatory activity (Figure 2). Even the bulky sugar substituent at the C8 position of bayin appears to inactivate the flavone (Redmond *et al.*, 1986). An apparent contradictory result was that apigenin-7-O-glucoside was found to induce good activity. However, examination of the purity of this commercial preparation by n.m.r. spectroscopy (data not shown) showed that it was contaminated with 3% apigenin (a strong inducer; see Table I).

The presence of a 3-hydroxyl group on the test compound (kaempferol, fisetin, quercetin, flavonol, morin and myricetin) also appeared to reduce the maximum inducible activity, although the number of compounds examined was limited.

Competition between inducer substances

Chrysin (Figure 2) and kaempferol caused significantly lower levels of induced *nod* gene transcription than 7,4′-dihydroxyflavone, but were active in a similar concentration range. It is possible that they bind to the same site as 7,4′-dihydroxyflavone. Evidence for such competition is provided by the results shown in Figure 3. The level of β-galactosidase activity induced by 5 × 10^{-7} M 7,4′-dihydroxyflavone is reduced by increasing concentrations of chrysin, reaching the limiting value that would be produced by chrysin alone. Similar results were obtained for competition between 7,4′-dihydroxyflavone and kaempferol.

We reasonsed that many of the compounds producing no stimulatory activity may similarly compete for binding sites and hence behave as inhibitors of the stimulatory activity of 7,4′-dihydroxyflavone. Evidence of this is given in Table II which

Table II. Measurements of the relative inhibitory ability of several flavones, isoflavones and coumarins

Inhibitor	Class of compound	Concentration causing 50% inhibition[a] (M)
chrysin	flavone	5×10^{-7}
kaempferol	flavanone	2×10^{-6}
formononetin	isoflavone	5×10^{-6}
diadzein	iosflavone	3×10^{-6}
biochanin A	isoflavone	$>5 \times 10^{-6}$
umbelliferone[b]	coumarin	4×10^{-7}

[a]The *nodA114* translation fusion derivative was used to generate the results in this table. Early log phase cells were exposed to 5×10^{-7} M 7,4′-dihydroxyflavone alone or in the presence of increasing concentrations of the inhibitory compounds. The concentration of inhibitory compound was recorded which caused a 50% reduction in the level of β-galactosidase activity; that is, to 50% of the level achieved with the exposure of cells to 7,4′-dihydroxyflavone alone. Induction times were 1.5 h.
[b]Addition of 5×10^{-6} M umbelliferone to cells exposed to 5×10^{-7} M 7,4′-dihydroxyflavone resulted in the total inhibition of β-galactosidase activity. Umbelliferone did not affect the expression of a *nodD* transcriptional fusion derivative (*nodD932*).

records the concentration of selective inhibitors required to give a 50% inhibition of the stimulatory effect of 5×10^{-7} M 7,4′-dihydroxyflavone (see Materials and methods).

All compounds that were able to compete with 5×10^{-7} M 7,4′-dihydroxyflavone and reduce the level of induced gene transcription to that due to the inhibitor substance, had molecular structures with a 7-hydroxy substituent. Note the structural differences and similarities between the flavones (many of which induce *nod* gene expression) and the isoflavones and coumarins (which usually inhibit *nod* gene expression) (Figure 2). None of the inhibitory compounds caused any detectable bacteriostatic effects at concentrations which resulted in inhibition of *nod* gene induction. The molecule with the strongest inhibitory effect was umbelliferone (7-hydroxycoumarin) (Figure 2). Unsubstituted coumarin produced only a small reduction in the amount of 7,4′-dihydroxyflavone-induced β-galactosidase activity. This behavior is consistent with the hypothesis that a 7-hydroxyl group is required for competitive binding. Only at high concentrations ($> 5 \times 10^{-6}$ M) did coumarin cause a small inhibitory effect on the growth of the cells. The definition of the structural requirements for inhibitor molecules, however, is made difficult by the possibilities for other independent inhibitory mechanisms and by the limited solubility in water of some of these compounds at concentrations $> 5 \times 10^{-6}$ M.

Identification of anti-inducers secreted by clover roots

In our previously reported studies on the release of stimulatory compounds from the roots of legumes (Redmond *et al.*, 1986), it was noted that a clear area was produced within the zone in which *nod* gene expression was stimulated. This was interpreted as evidence either for the release of substances which inhibit *nod* gene expression or for inhibition by higher concentrations of the inducers (Figure 4). Isolation of viable bacteria from the clear zone surrounding the root tip of clover plants was possible and this indicated that the inhibitory effect was not due to the presence of bactericidal agents.

Two inhibitory compounds, formononetin and umbelliferone, were previously isolated from clover root extracts (Redmond *et al.*, 1986), and these compounds were identified by comparison of their chromatographic behavior and n.m.r. spectra with those of authentic samples. Both were present in quantities greater than

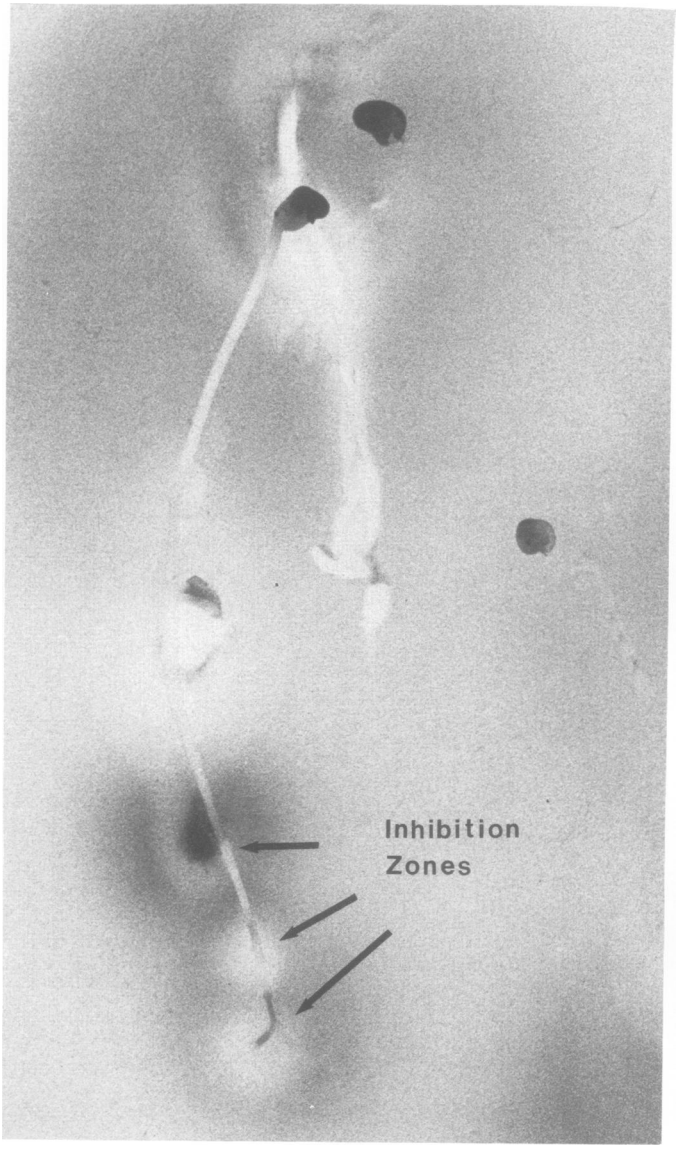

Fig. 4. Demonstration of the release of inhibitory compounds from the root tip of clover plants. White clover (and other legume plants; see Redmond *et al.*, 1986) release inhibitory (clear zones) and stimulatory (dark zone) compounds from the root tip area of undamaged plants which represses or induces *nodA* expression in the bacteria in the soft agar overlay surrounding the root tip. The expression of the *nodA* gene is indicated by the cleavage of X-gal which is incorporated into the soft agar overlay at 0.16 mg/ml. Note that the release of the inhibitory compounds appears to be cyclic as several distinct inhibitory zones can be clearly seen. The area surrounding the newly emerging root hairs is blue (dark area in the photograph), indicating that bacteria in this area are expressing their *nod* genes. Bacteria containing an anti-sense fusion to the *nodC* gene (*nod802*) do not show this result and neither do plants incubated on lawns without the appropriate bacteria, or bacteria incubated in the absence of the plant. Plant-derived β-galactosidase activity results in the plant root surface turning blue after several days and this rarely diffuses more than 1–2 mm into the overlay; blue zones caused by bacterial *nod* gene induction extend up to 15–20 mm into the soft-agar overlay after an overnight incubation.

those of the stimulatory flavones identified in white clover root exudates. The inhibitory effect of formononetin on *nod* gene expression is relatively weak, while the inhibitory effect of umbelliferone is stronger than all other compounds tested (Table II). A 10-fold molar excess of umbelliferone, relative to 7,4′-dihydroxyflavone, causes a complete inhibition of *nod* gene expres-

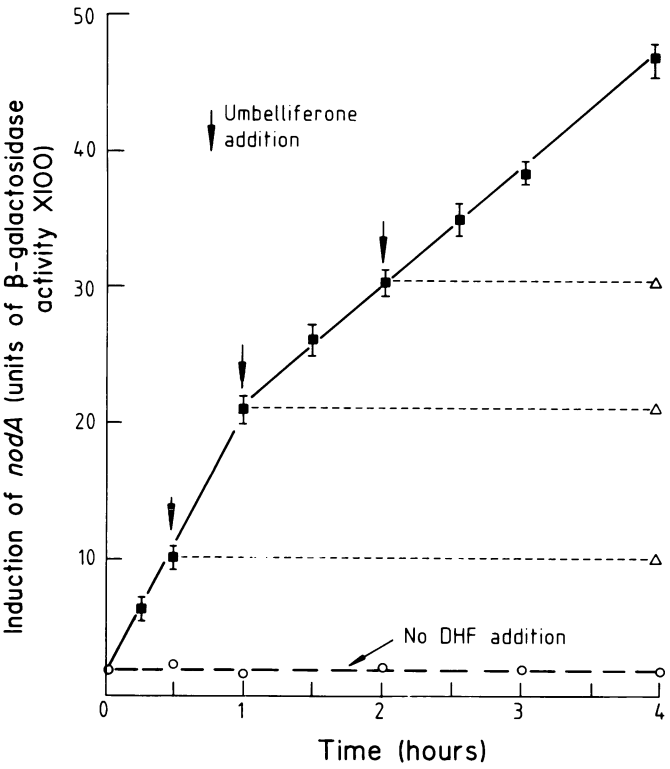

Fig. 5. Effect of umbelliferone additions to cells pre-exposed with 7,4′-dihdroxyflavone. This figure shows the response of a single batch of *R. trifolii* cells (*nodA* 218) incubated over a 4 h period (**a**) in the presence of 5×10^{-7} M 7,4′-dihydroxyflavone alone (■) or (**b**) in the presence of 7,4′-dihydroxyflavone for a period of time before a 10-fold molar excess of umbelliferone was added (△). The time points where umbelliferone was added are indicated by arrows. The addition of umbelliferone at the points shown results in the immediate cessation of the transcription of the *nodA* gene, as no further increase in the amount of β-galactosidase occurred. In experiments where the 10-fold molar excess of umbelliferone was used, the reactions were terminated at the 4 h mark. The simultaneous addition of 7,4′-dihydroxyflavone and a 10-fold molar excess of umbelliferone (at zero time) results in no detectable *nod* gene induction. About 200 units of background β-galactosidase activity was recorded in the absence of 7,4′-dihydroxyflavone (o).

sion (Figure 5). It therefore seems likely that umbelliferone secreted from undamaged clover cells in the vicinity of the root tip is the major cause of the 'inhibition zones' seen in Figure 4.

Rate of response of R. trifolii to signal compounds

R. trifolii cells containing a *nodA::lac* gene fusion were incubated with 5×10^{-7} M 7,4′-dihydroxyflavone for 15, 30, 60 and 120 min before the addition of a 10-fold molar excess of umbelliferone (Figure 5). This concentration of umbelliferone was sufficient to completely inhibit (>95%) the stimulatory effect of 7,4′-dihydroxyflavone. Measurable enhancement of β-galactosidase activity was observed within 20 min of the start of incubation and the level continued to rise until the umbelliferone was added. Addition of umbelliferone resulted in an immediate cessation of *nodA* gene transcription and the level of β-galactosidase activity remained constant for several hours (Figure 5). The addition of umbelliferone at concentrations which markedly inhibit *nod* gene induction by 7,4′-dihydroxyflavone had no detectable effect, either on the rate of growth of cultured cells, or on the amount of expression of *nodD* (data not shown).

Discussion

In *R. leguminosarum* expression of the *nodD* gene is constitutive but appears to be subject to autoregulation (Rossen *et al.*, 1985). Our results indicate that the expression of the *nodD* gene in *R. trifolii* is also constitutive, as previously reported (Innes *et al.*, 1985), but we also identify a growth period (early log phase) where the expression of the *nodD* gene per cell appears to increase substantially. The period of growth where enhanced inducibility of the *nodA* and *nodF* promoters occurs appears to mirror this transient increase in the amount of *nodD* expression. Cells outside this narrow growth window show a distinct inability to be induced by the addition of stimulatory flavones (at least for the 3 h induction periods used in this report). This could reflect several factors including poor uptake of the flavone compounds during the growth phases where induction is poor.

The dependence on growth phase of the amount of *nodD* gene product per cell is a finding of great importance for the performance of induction experiments. Since the *nodD* gene is actively transcribed during lag phase, the apparent rise in *nodD* expression per cell could reflect the transitory accumulation of this product before the cells begin dividing. If this is correct, then the length of the lag phase, which will also be a function of the time that the cells are kept in stationary phase before use, will greatly affect the sensitivity of the assay. For this reason, comparison of the ability of different substances to induce *nod* gene transcription should be done in parallel on the same batch of cells.

A consistent feature of both the stimulatory and inhibitory phenolic compounds is the presence of a 7-hydroxy moiety. This suggests that there is a common binding site for these molecules. Accordingly, the results presented here for competition assays between 7,4′-dihydroxyflavone and umbelliferone suggest that, (i) the displacement of 7,4′-dihydroxyflavone from the active site by umbelliferone is reversible and rapid; and (ii) displacement of 7,4′-dihydroxyflavone is accompanied by rapid loss of the stimulatory effect on gene transcription. In these experiments, β-galactosidase synthesised after translation of the fusion mRNAs is stable for at least 4 h. Similar conclusions can be drawn from the results presented for the inhibition of the transcription of the *nodA* gene by exposure of cells to chrysin (after a pre-exposure to set concentrations of 7,4′-dihydroxyflavone).

Firmin *et al.* (1986) have recently shown that the induction of *R. leguminosarum nod* genes by flavones (or the 7-glycosylated derivative of apigenin) can be inhibited by isoflavones such as diadzein and genistein. Their results are consistent with those presented here although 15 h exposures to the plant-derived compounds were used.

A model for *nod* gene activation has been proposed by Rossen *et al.* (1985). This model is similar to that proposed for regulation of the arabinose operon in *Escherichia coli* (Pabo and Sauer, 1984). Data presented here are consistent with this model if, as Rossen *et al.* (1985) speculate, the target site for the stimulatory and inhibitory compounds is the *nodD* gene product. Stimulatory compounds may mediate the conversion of *nodD* product to a positive regulatory form while inhibitory compounds may compete for and occupy the binding site resulting in only poor or no 'activation' of the *nodD* product. The activated *nodD* product may stimulate transcription from the conserved nod-box sequences which occur 5′ to several inducible *nod* operons (Rostas *et al.*, 1986; Schofield and Watson, 1986; Scott 1986; Shearman *et al.*, 1986).

However, one must consider that the uptake of hydrophobic molecules by most Gram-negative bacteria (particularly soil microorganisms) is reported to be very inefficient (Hancock, 1984 and references therein). Thus the rapidity of the response seen after addition of these phenolic compounds at low concentrations suggests that either these compounds traverse the bacterial cell

wall or perhaps there is some involvement of an active, membrane-bound uptake or transmembrane signally system. With the data presented thus far, one cannot rule out that these phenolic compounds act at a common membrane site and that a secondary signal is responsible for the induction of the *nod* genes, either in combination with the *nodD* product or perhaps by activating the *nodD* product itself. The membrane-located *virA* gene product of *Agrobacterium tumefaciens* is reported to act in this way, responding to the presence of acetosyringone and similar phenolic compounds in the environment, by activating the *virG* product (Winans *et al.*, 1986).

The high concentrations of inhibitory compounds at the root tip of clover and of stimulatory compounds at or near the zone of emerging root hairs is consistent with the biology of infectivity shown for several legumes (Bhuvaneswari *et al.*, 1980; Ridge and Rolfe, 1985). The root hairs of many legume plants are transiently infectible (Bhuvaneswari *et al.*, 1980) and so the rapid response to the presence of the stimulatory flavone is probably a necessary adaption in order to maximise the possibility of infection by the bacterium of the highly-infectible, pre-emergent root hair cells. Legumes may need to protect the rapidly dividing plant cells that occur between the emerging root hairs and the root tip from rhizobia that may degrade their more delicate cell walls at a developmentally sensitive stage and this may be the reason why high concentrations of the inhibitory compounds are released from this area of the plant (Figure 4). The fluctuations in the concentration of the inhibitory compounds (seen in Figure 4) may also at least partially explain the variations in the intensity of the root hair curling reaction and infectivity often seen on legume roots, particularly if the concentrations of stimulatory and inhibitory compounds within the plant root change during the infection process. The identification of the stimulatory and inhibitory plant compounds will allow bacterial mutants to be isolated which are no longer sensitive to the inhibitory compounds, or which react to a limited range of flavone compounds. This, in turn, should permit an understanding of the role of these compounds in bacterial competition for transiently infectible root hair cells. It is also possible that the difference between more competitive and less competitive *Rhizobium* strains is that competitive strains either: (i) respond favourably to lower concentrations of the stimulatory flavones; (ii) respond to a different range of flavones; (iii) respond more rapidly to the same flavones; or (iv) they are less sensitive to the inhibitory compounds secreted by legumes. It is yet to be determined if specific pairings of flavones/isoflavones/coumarins and *nodD* gene products have a role in competition interactions.

Materials and methods

Bacterial strains

Most of the bacterial strains used were derivatives of the wild-type *R.trifolii* strain ANU843 in which the symbiotic plasmid had been replaced by plasmids derived from pRt032. Plasmid pRt032 contains the 14-kb region carrying the nodulation genes from the *R. trifolii* symbiotic plasmid (Schofield *et al.*, 1984). Only derivatives of pRt032 containing either translational or transcriptional fusions of the *E. coli lac* operon induced by either Mud*I* 1734 or Mud*II* 1734 (Castilho *et al.*, 1984; Innes *et al.*, 1985) were used in this study. These were pRt032::*nod*218 (*nodA*), pRt032;;*nod*1027 (*nodFE*), and pRt032::*nod*932 (*nodD*), described previously (Innes *et al.*, 1985) and pRt032::*nod*114, (which contains a translational fusion of Mud*II* 1734 to the *nodA* gene). The translational fusion derivative of plasmid pRt032 was isolated as previously described for transcriptional fusions (Innes *et al.*, 1985). Plasmid pRt032::*nod*802, which contains an anti-sense fusion of Mud*I* 1734 to the *nodC* gene, was used as a negative control.

Assays of nod gene induction and expression

In vivo assays showing the expression of *nod* genes in bacteria near plant roots were performed as previously described (Redmond *et al.*, 1986). Induction of *nod* gene expression was indicated by the blue colour produced by β-galactosidase activity using 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) at 0.16 mg/ml as substrate.

The activity of β-galactosidase generated by bacteria grown in solution was measured using the method reported by Miller (1972) except that the cells were incubated at 42°C for 30 min before the addition of the *o*-nitrophenyl-β-D-galactopyranoside substrate to deactivate native *R. trifolii* β-galactosidase. *E. coli* β-galactosidase activity is resistant to incubations at 42°C (R.Okker, personal communication). Under the growth conditions used, the background activity of native bacterial β-galactosidase was minimal and did not interfere with estimations of *nod* gene induction.

The activity of *nod* genes throughout the growth cycle of various mutants was assessed on bacteria grown in BMM medium (Rolfe *et al.*, 1980). Bacteria, grown overnight to log phase were diluted into fresh BMM medium and samples were taken subsequently every 4 h. Half the aliquot (400 μl) was lysed immediately and the 'background' activity of β-galactosidase was determined. The other half was first incubated at 29°C for 3 h with 5×10^{-7} M 7,4′-dihydroxyflavone and then lysed and examined for β-galactosidase activity.

Inhibition assays

The effect of inhibitory compounds was assessed by determining the concentration required for reduction of *nod* gene transcription in the presence of 5×10^{-7} M 7,4′-dihydroxyflavone to 50% of the value in the absence of inhibitor. Where compounds had both (weak) stimulatory and inhibitory activity in the presence of 7,4′-dihydroxyflavone, the concentration of this compound which gave 50% inhibition was defined as follows: the concentration of the inhibitor which gave a level of β-galactosidase activity which was half-way between the level of induction achieved in the presence of 7,4′hydroxyflavone alone and the level achieved in the presence of the inhibitor alone (see Figure 3).

Isolation of phenolic compounds from white clover root exudates

Umbelliferone and formononetin were previously identified by n.m.r. spectroscopy as being present in root washings of germinated, undamaged clover seedlings (Redmond *et al.*, 1986). The isolation procedure has been described (Redmond *et al.*, 1986). Commercial preparations of umbelliferone and formononetin were used in this study.

Chemicals

Synthetic 7,4′-dihydroxyflavone and Bayin were obtained as gifts from Professor R.Eade and Professor J.Stevens of the University of New South Wales. Geraldone and geraldone chalcone were synthesised by J.W.R. The authenticity and purity of these compounds was checked by proton n.m.r. spectroscopy.

Chrysin, apigenin, naringenin, kaempferol, quercetin, taxifolin, flavone, morin, myricetin, *o*-, *p*- and *m*-benzoic acid, *p*-hydroxybenzoic acid, 5,7-dihydroxyflavone umbelliferone and naringin were all purchased from Sigma Chemical Co., St Louis, MO, USA. Fiesetin, flavonol, 2,4-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid and diosmin were purchased from Aldrich Chemical Company, Milwaukee, WI, USA; 7-hydroxyflavone, diadzein and formononetin from ICN Biomedicals, Plainview, NY, USA; luteolin, apiin and apigenin monoglucoside from Research Plus, NY, USA; coumarin and *p*-hydroxybenzoic acid from Fluka Chemical Co., Switzerland; 3,4-dihydroxybenzoic acid from Koch-Light; and 3,5-dihydroxybenzoic acid from Merck, Darmstadt, FRG.

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