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Mini Review

Plant Flavonoids: Signals to Legume Nodulation and Soil Microorganisms

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Flavonoids released from plants play an important role as signal molecules in early stages of legume-*Rhizobium* symbiosis and in regulating the activities of soil microorganisms. Development of an assay system for the detection of plant-derived stimulatory compounds has led to isolation and identification of compounds responsible for activation of nodulation (*nod*) genes in different legumes. In addition to flavonoids, betaines (in alfalfa) and chalcones (in vetch) have been identified as *nod* inducers. Seeds and roots of a legume release structurally different *nod* inducing flavonoids. These hydroxylated flavonoid compounds are derived from phenylpropanoid pathway which are responsible for synthesis of many important plant phenolic compounds, including phytoalexin molecules, which are thought to be involved in plant defence system. Flavonoids not only regulate transcription of rhizobial *nod* genes, but also promote chemotaxis, growth, metabolism and symbiotic efficiency of rhizobia. Important effects on other soil microorganisms are evident as some flavonoids in seed and root exudates promote spore germination in mycorrhizal fungi. Knowledge derived from studies from how plants regulate rhizobia and other soil microorganisms with natural plant products offers a basis of defining new concepts of rhizosphere ecology.

Key words: flavonoids, legume, microbial growth, nodulation, *Rhizobium*, root exudate, symbiosis.

Flavonoids play an important role as signal molecules in the early stage of legume- *Rhizobium* symbiosis. Flavonoids exuded by plant root, induce in a host specific way, transcription of nodulation (*nod, noi, nol*) genes in *Rhizobium, Bradyrhizobium,* and *Azorhizobium* (collectively referred here as rhizobia) by activating NodD protein produced from regulatory *nod* D genes and initiate nodule development on legume plant. Flavonoid activated *nod* genes of these rhizobial species encode enzymes involved in the formation of acylated and sulphated lipo-chitooligosaccharide (LCO) signal molecules (1) which trigger root hair deformation and curling, infection and cortical cell division leading to nodule formation (Fig. 1). Some of these compounds play a broader role by inducing growth (2,3) and positive chemotaxis in rhizobia (4).

Traces of flavonoids in root exudate were first reported in 1944 in wheat (5) and later in many legumes (6), but accurate identification and quantification of flavonoids was done in 1986 in root exudates of soybean and lentil (7). Subsequent discovery that some flavonoids functioned as transcriptional signals in rhizobia emphasizes the importance of initial observation and is the main subject of this review. Understanding how plants release flavonoid signals that affect microbe, is the first step towards managing these molecules in agricultural and natural ecosystem.

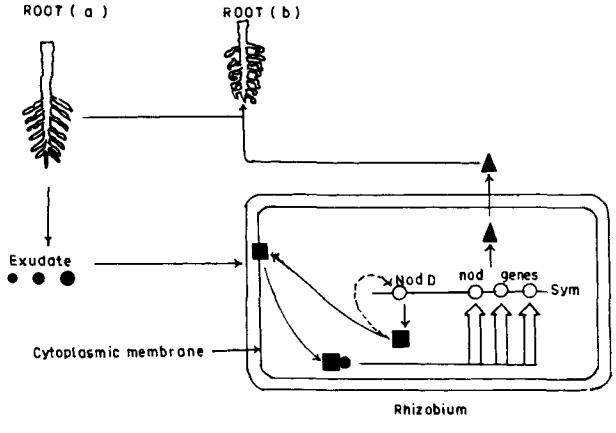

Fig. 1. Schematic representation of induction of *nod* genes: ● Nod regulators; ■ NodD protein; o Promoter; ▲ Nod factor; ⇒ Induction; ⇢ Repression.

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Exudation of the *nod* Gene Inducing Molecules by Host Plants

Studies with legume root exudates — As early as in 1929, the effect of legume root exudate was studied on *Rhizobium*-legume symbiosis. The root exudates were reported to both stimulate (8) and inhibit (9) nodulation by rhizobia. The substance regulating nodulation was not then identified, however, was found to affect early stage of nodulation such as chemotaxis (10), adhesion (11) adsorption of *Rhizobium* to its host roots (12). In addition, it was reported that cowpea root exudate enhanced nodulation of host by *Rhizobium* (13). Nitrogenase activity of chickpea-*Rhizobium* association was also elevated by root exudates obtained from young seedlings of *Cicer arietinum* (14).

Detection of inducing compounds from plants: the flavonoids — Several research groups showed that in addition to regulatory *nod* D gene, transcription of *nod* genes required an unknown plant factor (15). These results were based on the use of reporter gene *lac* Z. Several flavonoids were identified as active rhizobial *nod* inducing plant signal molecules from host root exudates and extracts (Table 1). Luteolin (5,7,3′4′-tetrahydroxy flavone) from alfalfa and 7,4′-dihydroxyflavone from white clover were identified as *nod* gene inducers in *R. meliloti* (16) and *R. leguminosarum* bv. *trifolii* (17) respectively. From pea, apigenin-7-O-glucoside (5,7,4′-trihydroxy flavone-7-O-glucoside) and eriodictyol (5,7,3′,4′-tetrahydroxyflavanone) were implicated as *nod* gene inducers for *R. leguminosarum bv. viciae* (18,19), while daidzein (7,4′-dihydroxyisoflavone) and genistein (5,7,4′-trihydroxyisoflavone) were identified from soybean as inducers in *B. japonicum* (20). Subsequently *nod* genes inducing compounds were identified in *Vicia sativa* sub species *nigra* (vetch) (15), *Trifolium pratense* (21), *Phaseolus* (22) and *Lotus* species (23,24). In general, flavones and flavanones were identified as rhizobial *nod* genes inducing molecules in seeds, root exudates and extracts from temperate legumes. For tropical legumes such as soybeans and common beans, the isoflavones were identified as main inducing substances in the cotyledons and root exudates. In addition to flavonoids, chalcones (25,26), anthocynidins (22), betaines (27) and conjugated isoflavonoids (23) were identified as having *nod* gene inducing activity (Fig. 2). These compounds are derived from the phenylpropanoid pathway (28). The *nod* inducing compounds are active at very low concentrations and are shown to stimulate *nod* gene expression within minutes of their interaction with rhizobia (15).

Table 1. The *nod* gene inducers released by different legumes

Source	Inducers	Reference
Soybean seed exudate	Genistin; genistein-7-O-(6'-O-malonylglucoside); daidzein-7-O-(6'-O-malonylglucoside); daidzein-2-O-glucoside with undefined acetylation	90, 30
Soybean root exudate	Isoliquiritigenin	31
Alfalfa root exudate	7, 4'-dihydroxyflavone; 4, 4'-dihydroxy-2'-methoxy chalcone; liquiritigenin; formononetin; formononetin-7-O (6'-O malonyglycoside); formononetin-7-O glycoside	22, 25, 27, 51
Alfalfa seed exudate	Luteolin; chrysoeriol; 5, 3'-dimethoxyluteolin; 5-methoxyluteolin; trigonelline; stachydrine	16, 27, 35
Common bean root exudate	Genistein; genistein-7-O-glycoside; eriodictyol; naringenin; daidzein; coumestrol; liquiritigenin; isoliquiritigenin	22, 32, 33
Common bean seed exudate	Delphinindin-3-O-glycosides; petunidin-3-O-glycosides; malvidin-3-O-glycosides; myricetin-3-O-glycosides; quercetin-3-O-glycosides; kaempferol-3-O-glycosides	32
Vetch root exudate	3, 5, 7, 3'-tetrahydroxy-4'-methoxyflavanone; 7, 3'-dihydroxy-4'-methoxy flavanone; 4, 2', 4'-trihydroxychalcone; 4, 4'-dihydroxy-2'-methoxychalcone; naringenin; liquiritigenin; 7, 4'-dihydroxy-3'-methoxyflavanone; 5, 7, 4'-trihydroxy-3'-methoxy flavanone; 5, 7, 4' trihydroxy-3'-methoxyflavanone; 5, 7, 3'-trihydroxy-4'-methoxyflavanone	15, 26

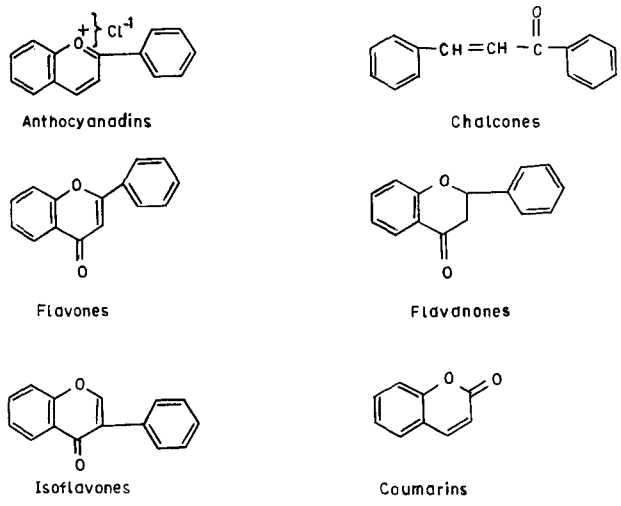

Fig. 2. Structure of some of the *nod* inducing flavonoids.

Soybean produces two major groups of *nod* gene inducing isoflavonoids, the 5-hydroxy series, based on genistein and 5-deoxy series of daidzein conjugates. Both compounds are released from roots and cotyledons of germinating seeds as 6′-O-malonyl-7-glucosides (29), and these conjugates show *nod* gene inducing activity (30). Isoliquiritigenin, a strong *nod* inducing chalcone, was identified as being present only in root exudate and not in seed rinse (31).

Alfalfa has been found to have a higher rhizobial species specificity for *nod* gene induction than other legumes studied so far. Roots do not release luteolin, the first alfalfa *nod* gene inducer identified (16), but they do release three other *nod* gene inducing flavonoids (25). The 4,4′-dihydroxy-2′-methoxychalcone (Mch) released by roots induced transcription of *nodC-lacZ* fusions in *R. meliloti* at a ten times lower concentration than luteolin (25) and gives an apparent activation of both NodD1 and NodD2 proteins. The 7,4′-dihydroxyflavone and liquiritigenin (7,4′-dihydroxyflavanone) induce *nod* C-*lac* Z fusions at concentration similar to luteolin. Like Mch, isoflavone formononetin-7-O-malonylglycoside (FMG) exuded by root, activated both NodD1 and NodD2 proteins. Alfalfa seeds release two major flavonoids *nod* gene inducers, luteolin and chrysoeriol (3′-methoxyluteolin)(31). Chrysoeriol has about 60% lower I_{50} value than luteolin, thus methoxylation at 3′-OH group by alfalfa probably enhances plant-microbe interaction. In addition to numerous *nod* gene inducing flavonoids, alfalfa seeds also release two betaines, stachydrine and trigonelline, which apparently induce transcription of *nod*C-*lac* Z fusions in *R. meliloti* by activating NodD2 protein (27).

Seeds and roots of common bean release a wide variety of *nod* gene inducers including anthocynidins, flavonols, isoflavonoids and flavanones (32,33). Eight *nod* gene inducing flavonoids, daidzein, genistein (22), naringenin, eridictyol, genistein-7-O-glycoside (32), isoliquiritigenin, coumestrol and liquiritigenin were identified in root exudate. The seed-rinse from the common beans has been found to contain 3-O-glycosides of delphinidin, petunidin, malvidin, myricetin, quercetin and kaempferol (22). Despite the diversity of signals available from the host plant, three different copies of regulatory *nod* D gene: *nod* D1, *nod* D2 and *nod* D3 showed essentially no specificity to the inducer molecule.

Root exudates of vetch contained a series of *nod* inducing flavonoids including six flavanones (liquiritigenin, 7,4′-dihydroxy 3′-methoxyflavanone, 7, 3′-dihydroxy-4′-methoxyflavanone, naringenin, homoeriodictyol, hesperitin and two chalcones (Mch and isoliquiritigenin). These were exuded in the presence *of R. leguminosarum* bv. *viciae* (26). *Vicia faba* (faba bean) released 7,4′-dihydroxyflavone, 7, 3′,4′-trihydroxyflavone and a 7-O-glycoside of quercetin and a 7-O-glycoside of kaempferol (34). These flavonoids are released as aglycones or glycosidic conjugates. The latter are less active but have a high solubility in water and are converted into active form (aglycone) by bacterial glycosidases (22).

Effect of structural variations in the flavonoids released from different legumes on *nod* gene induction was confirmed by cloning *nod* D gene of different species of rhizobia into a *Rhizobium* strain lacking its normal symbiotic plasmid. The induction of the *nod*ABC genes in cloned rhizobia was highest in the presence of flavonoids found in the normal host (Table 2). It was also confirmed by the fact that *nod* D gene product from a strain of *Rhizobium* that nodulate siratro (*Macroptilium atropurpureum*) but not alfalfa, was activated by siratro seed flavonoids after being transferred to *R. meliloti* (16). Inoculation of host legume with compatible *Rhizobium* has been reported to stimulate a release of both *nod* gene inducers and other flavonoids (22,33). Whether flavonoids released in response to rhizobia are produced by new synthesis or by hydrolysis of stored conjugates remain to be confirmed.

Regulation of plant flavonoids release — Methanol removed large quantities of flavonoids from seeds without producing normal hydration events associated with germination (35). Thus flavonoids deposited on seed coat during seed formation were rinsed off during imbibition without any regulatory controls through metabolic processes. In contrast, the storage and release of

Table 2. Effect of flavonoids and chalcones in conjuction with various Nod D proteins on *nod* gene expression

Compound	Substitution Position						Nod D Protein					
	3	5	7	3′	4′	5′	Rt	Rl	Rm D1	Rm D2	NGR 234	Bj
Flavones												
Luteolin		OH	OH	OH	OH		++	++	++	-	++	-
Apigenin		OH	OH		OH		++	++	+	-	++	+
3′, 4′, 7-Trihydroxy flavone			OH	OH	OH		nd	++	++	-	nd	nd
3′, 4′-Dihydroxy flavone				OH	OH		nd	nd	-	-	nd	nd
Chrysoeriol		OH	OH	OCH_3	OH		nd	nd	++	-	nd	nd
Chrysin		OH	OH				+	-	-	-	++	-
Flavonols												
Myricetin	OH	OH	OH	OH	OH	OH	-	-	-	-	+	nd
Quercetin	OH	OH	OH	OH	OH		-	-	-	-	++	-
Kaempfrol	OH	OH	OH		OH		-	-	-	-	++	+
Flavanones												
Eriodictyol		OH	OH	OH	OH		+	++	+	-	nd	-
Naringenin		OH	OH		OH		++	++	-	-	++	-
Hesperitin		OH	OH	OH	OCH_3		-	++	-	-	++	nd
Isoflavones												
Genistein		OH	OH		OH		-	-	-	-	++	++
Daidzein			OH		OH		-	-	-	-	++	++
Chalcone												
4, 4'-Dihydroxy-2'-methoxychalcone			OH		OH		nd	nd	++	++	nd	nd

Note : Rt, Rl, Rm, NGR, and Bj refers to *R. trifolii, R. leguminosarum* bv *viciae, R. meliloti, Rhizobium* sp. NGR 234 and *B. japonicum,* respectively.
nod gene expression- ++ : >50%; + 10 - 50%; - : <10%; nd: not determined.

flavonoid *nod* gene inducers in roots are under control. Roots of alfalfa seedlings and many other legumes contained conjugated form of isoflavonoid formononetin (36) and the infection of plant by *R. meliloti* promoted (31) release of a formononetin-7-O-(6′-O-malonylglycoside). Maxwell and Phillips (36) showed that labeled (U-^{14}C) phenylalanine incorporated into flavonoid was exuded from the roots. In the presence of phenylalanine ammonia lyase (PAL) inhibitor, 2-aminooxy-3-phenylpropionic acid (AOPP), the synthesis of inducer decreased by 90-95% but the exudation of 7,4′-dihydroxyflavanone was only 50% inhibited (37). Presence of high amount of unlabelled 7,4′-dihydroxyflavanone in the root exudate from seedling treated with AOPP indicated that the compound could be exuded from reserve pool within the root. Exudation of other *nod* gene inducers such as 7,4′-dihydroxyflavone and 4,4′-dihydroxy 2′-methoxychalcone was tightly linked to concurrent synthesis. Zon and Amrhein (38) showed that inhibition of PAL activity using 2-aminoindan-2-phosphonic acid (AIP) markedly decreased nodulation of alfalfa roots.

Generation of Flavonoid Signals to Rhizobia

Synthesis — The flavonoids are products of branched pathway originating from the central phenylpropanoid pathway and the acetate malonate pathway (28). Thus all flavonoids are derived from phenylalanine produced from the shikimic acid pathway and malonyl CoA originating via acetyl CoA carboxylase reaction. Condensation of 4-coumaroyl CoA and three molecules of of malonyl CoA by chalcone synthase (CHS) leads to the production of flavonoids (Fig. 3). Being the first enzyme in flavonoid pathway, CHS is an important regulatory enzyme. Initial molecule formed by chalcone synthase is a chalcone hydroxylated at C-4,4′,6′ of the chalcone or the corresponding C-4′,5,7 positions in the flavone. Biosynthetic relationship among flavonoid *nod* gene inducers reported from alfalfa reflects a dichotomy between the 5-hydroxyluteolin-chrysoeriol pathway and 5-deoxy compounds found in root exudates. In *Glycyrrhiza* (32) and soybean (39), this difference is produced by polyketide reductase (trivial name chalcone reductase, CHR) which reduces the corresponding carbonyl group

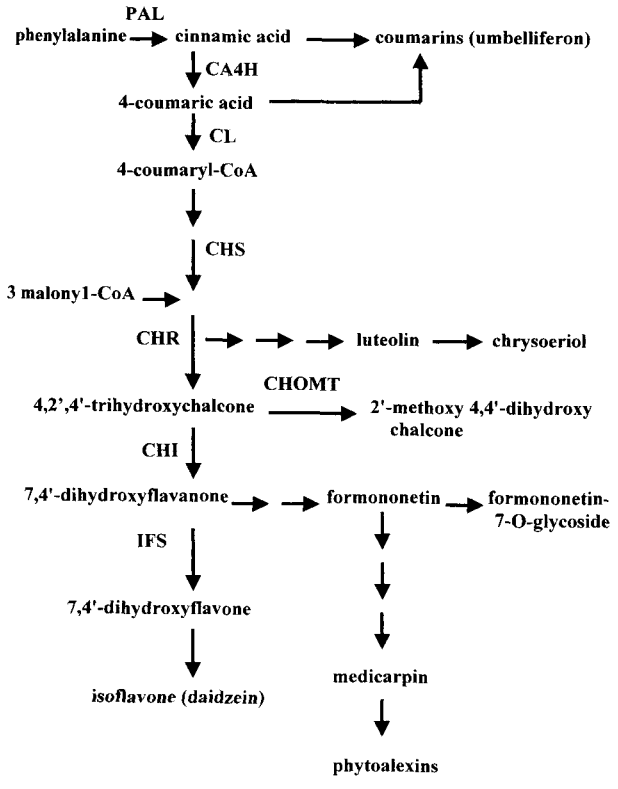

Fig. 3. Proposed phenylpropanoid pathway for biosynthesis of flavonoids and isoflavonoids. The flavonoids induce fast growing rhizobia *R. meliloti*, *R. leguminosarum* while the isoflavonoids induce the fast growing strain NGR 234 and slow growing *Bradyrhizobium* strains. PAL, phenylalanine ammonia lyase; CA4H, cinnamic acid 4-hydroxylase; CL, coumaroylCoA ligase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; CHOMT, chalcone 2'-O-methyl transferase; IFS, isoflavone synthase.

on the polyketide prior to cyclization of aromatic A ring with subsequent loss of the oxygen as water (40). It functions with CHS to eliminate 6-hydroxyl group on the resulting chalcone. Substrate for CHR is therefore the polyketide intermediate bound to the condensing / cyclizing enzyme CHS. Ballance and Dixon (41) isolated chalcone reductase cDNA clones from alfalfa and demonstrated co-ordinate transcriptional activation of CHR and CHS. Product of co-action of CHS and CHR, 2',4,4-trihyroxychalcone is a branch point metabolite for synthesis of flavonoids in legumes with distinctly different biological activities. Chalcone isomerase (CHI) converts the chalcone to 7, 4'-dihydroxyflavanone (liquiritigenin). In contrast, chalcone-2'-O-methyl transferase (CHOMT) catalyzes the formation of 2'-methoxy-4,4'-dihydroxychalcone, the most potent inducer of *Rhizobium* nodulation genes released from alfalfa roots (25). This is a key signal metabolite for initiation of *Rhizobium*-alfalfa symbiosis.

Regulation — Presence of rhizobia is reported to be a major factor that affects production of *nod* gene inducing molecules in several legumes (22,33). *Rhizobium* dependent increase in *nod* gene inducing activity in the extract of white clover (42), vetch (26,), soybean (43), alfalfa (44) and common bean (33) has indicated that the presence of rhizobia affected production and release of *nod* gene inducing flavonoids in the host root exudate. Elicitors from rhizobia may also initiate flavonoid production. Role of *nod* factors in eliciting additional flavonoid production from *Vicia* was described by Spaink (45).

Molecular biology evidences indicate that rhizobia regulate transcription of chalcone synthase (CHS), a key enzyme for flavonoid biosynthesis in legumes. At least seven different CHS isoforms have been characterized in alfalfa (46) and six in *P. vulgaris* (47). Inoculation by an infective *R. leguminosarum* bv. *viciae* strain on *Vicia* increased CHS mRNA, while non-infective rhizobia had no effect (26). CHS promoter contained *cis* acting sequences that functioned as both activator and silencers in presence of *trans* acting factors, and exogenous products from phenylpropanoid pathway affected the regulation. It is not known whether functional differences exist between different CHS isoforms in legumes. Evidence from soybean suggests that symbiotic and pathogenic microbes may affect different chalcone synthase and phenylalanine ammonia lyase promoters (48). These results support the concept that external biological factors like symbionts may alter flavonoid synthesis through molecular effects on key promoters.

Transmission — Transmission of flavonoids through soil helps in understanding the regulation of microbial genes by *nod* inducing flavonoids that control legume root colonization and nodulation. Simple phenolic compounds and flavonoids having *nod* gene inducing activity can be easily extracted from the soil around alfalfa plants (49). Decreasing concentration of *nod* gene inducing activity extracted from soil with increasing distance from rhizosphere supported the concept that alfalfa roots were the source of *nod* inducing compounds.

Flavonoids exuded in soil as glycosides are hydrolyzed by glycosidases released from germinating host seedlings and the sugar released is utilized for energy (2). Not all flavonoids are degraded in soil. Medicarpin has been reported to accumulate in alfalfa-rhizosphere and inhibit seed germination and seedling growth (50). Absence of detectable amount of 4,4'-dihydroxy-

2'methoxychalcone, 4'7-dihydroxyflavanone or 4',7-dihydroflavone in the extracts from alfalfa-rhizosphere soil (51) is an indirect evidence for degradation of these alfalfa *nod* gene inducers, but the mechanism/agent involved in their catabolism is not known.

Role of Flavonoids in Nodulation

Induction of nod genes — Expression of *nod* genes is positively regulated by LysR- type trans-activator protein NodD in the presence of host plant derived flavonoid(s) (52). Flavonoid inducible *nod* operons are preceded by a conserved *cis* regulatory element, the *nod* box. NodD binds to *nod* box sequence and upon interaction with inducing flavonoid, activates transcription of *nod* operons. Specific flavonoids interact with *nod* D genes from specific rhizobial species and this represents the first level of host specific recognition.

Using synthetic or natural compounds of known structure, the structural features of flavonoids necessary for *nod* gene induction by different NodD proteins have been determined (Table 2). NodD proteins are generally active in the presence of a family of related flavonoids. NodDs from fastidious rhizobia (*R. meliloti*, *R. leguminosarum*, *R. trifolii*) respond to a few flavonoids, while NodDs from promiscuous *Rhizobium* NGR 234 has a large spectrum of flavonoids including monocyclic aromatic compounds (53) (Table 2). The activity of a given flavonoid compound vari es widely with the rhizobial species. Induction of *nod* genes by flavonoid(s) leads to the production and secretion of return signals, the Nod factors which are lipochito-oligosaccharide (LCO) of variable structures, depending on the host plant and subset of *nod* genes that each induces (54). Each *Rhizobium*-strain produces a characteristic pattern of Nod factors (55) that is usually unique for a given isolate though basic structure (LCO) of Nod factor is common to all rhizobia. Nod factor profile seems to reflect adaptation to the host plant. Thus, rhizobia belonging to different taxonomic groups produce LCOs of similar structure when isolated from the same host plant (56).

In addition to the basic activation mechanism of *nod* genes by NodD protein upon interaction with specific flavonoids of host plants, there are number of additional control circuits. Many species of rhizobia contain several generally highly homologous *nod* D genes (32) that result in production of NodD proteins capable of interacting with a range of different inducer molecules. There may be additional *nod* gene activators, for example a two component regulatory system with *nod* V as a flavonoid sensor and *nod* W as regulator has been identified in *Bradyrhizobium japonicum* (57). NodV has a different inducer spectrum than NodD and this appears to widen the host range (52).

Anti-inducers of nod genes — The seeds/roots of legume plants also secrete flavonoids which antagonize the flavonoid mediated activation of *nod* genes (Table 3). In several cases flavonoids devoid of *nod* gene inducing property inhibit *nod* gene activation by effective inducers (18,58-60). Anti-inducers generally have structures similar to those of inducers and inhibition can be overcome by increasing the concentration of inducers, hence are considered as competitive inhibitors (58). Some of these inhibitors are strain specific, acting on a few strains belonging to the same cross-inoculation group (60). Chrysin, kaempferol, daidzein, genistein and acetophenone analogues inhibited the induction of *nod* genes of *R. leguminosarum* bv. *viciae* (18). It is interesting that isoflavones (daidzein and genistein) act as inducer molecules for *nod* gene expression in *Bradyrhizobium* strains and the fast growing *Rhizobium* strain NGR 234 but are anti-inducers for most narrow host range rhizobia of clover and pea. Some *nod* gene inducing flavonoids also act as partial inhibitors of *nod* induction in a particular environment. Chrysin and kaempferol caused lower level of induction of *nod genes* of *R. trifolii* than 7,4'-dihydroxyflavone (DHF) in a similar concentration range.

Table 3. Major antagonists of inducers of rhizobial and bradyrhizobial *nod* genes

Symbiotic association	Antagonist compounds	Reference
R. leguminosarum bv. *trifolii* - clover	Umbelliferone; formononetin	59
R. leguminosarum bv. *viciae* - pea	Daidzein; genistein; kaempferol; acetovanollone; syringaldehyde	18
R. leguminosarum bv. *phaseoli* - common bean	Umbelliferone; acetosyringone	60
R. meliloti - alfalfa	Umbelliferone; morin; quercetin	58
B. japonicum - soybean	7-hydroxy-5-methyl flavone; flavone; kaempferol; chrysin	60

But when these molecules were tested in competition experiments with stimulatory DHF, they inhibited *nod* gene induction (59). Similarly, isoliquiritigenin, a weak inducer of *B. japonicum nod* genes antagonized the induction by daidzein when both were applied in combination. Spatio-temporal distribution of flavonoids in rhizosphere and at root surface is likely to determine the levels of induction of rhizobial *nod* genes. The chief determinant of nodule initiation site might therefore be the ratio of stimulator to inhibitor in the vicinity of potential infection site or inside the induced infection thread.

Enhancing symbiotic efficiency — Flavonoid induced synthesis of Nod factors by rhizobial *nod* genes is responsible for initiating a cascade of events in the host plant that leads to nodule organogenesis. Nod factors trigger root hair deformation and curling (61), cortical cell division (4) and expression of a class of nodule specific plant genes, the early nodulins (62) and induction of root primordia (55). Root hairs respond to Nod factors with a rapid transient plasma membrane depolarization (63) with Ca^{2+} influx and Cl^- and K^+ efflux (80). The change in intracellular Ca^{2+} concentration triggers a further signal transduction cascade that ends up in the expression of early nodulin genes *Enod* 12 (65) and *rip* 1 and production of ENOD 12 and RIP 1 proteins in nodule primordia as well in root hair (66). In *Medicago sativa* two *Enod* 12 genes have been identified that differed in temporal and spatial expression pattern suggesting a role of *Enod* 12 at various stages of nodule development (67). The role of flavonoids has also been reported in nodule functioning as leghaemoglobin expression has been shown to be induced by Nod factors even prior to early nodulins (68). Other genes involved in nodulation signaling like protein kinases (69), cell cycle control (70) cd c_2 gene and cyclin genes (71,72) have been reported to function as auxin transport inhibitors (71), thereby inducing nodule like structure on the host plant. It has been suggested that flavonoids/ Nod factors induced cell division might be mediated by a perturbation of auxin flow (73).

Rhizospheric application of flavonoids increased nodulation and nitrogen fixation in pea, alfalfa and mungbean under controlled conditions. In alfalfa, luteolin and naringenin is reported to have a positive effect (74,75). In mungbean syringaldehyde (76) and in pea naringenin (77) enhanced nodulation and nitrogen fixation. Presumably, these results indicate that under conditions of experiments flavonoids levels released from roots and seeds may be limiting. Thus, addition of flavonoids may have enhanced nodulation by promoting growth of bacteria and by induction of *nod* genes. Nitrate is known to inhibit legume root nodulation and reduce flavonoid concentration in the root exudate (43) Inhibition of nodulation by nitrate is partially overcome by application of naringenin in pea (78).

Effect of Flavonoids on Microorganisms

In addition to nodulation, flavonoids have been reported to affect other microbial processes also. Flavonoids exuded by root act as plant signal to microbes. Microbial responses to these nano to micromolar concentrations of flavonoids represent reactions that generally increase the potential for subsequent plant-microbe contact. Major actions of flavonoids include effect on chemotaxis, growth and metabolism and development of bacteria and fungi.

Chemotaxis — Flavonoids present in the root exudate influence rhizobial infection by mechanisms additional to *nod* gene regulation. Rhizobia respond by positive chemotaxis to plant root exudates leading to their movement towards localized sites on legume roots (4,79,80). Some flavonoids show specificity for chemotaxis similar to *nod* inducing species specificity. In *R. leguminosarum* bv. *phaseoli*, apigenin and luteolin acted as strong chemoattractants but naringenin had no effect (79). *R. leguminosarum* bv. *viciae* moved towards naringenin, kaempferol and apigenin but hesperitin produced no chemotaxis (80). *R. meliloti* showed positive chemotactic response to luteolin, but did not respond either to apigenin or naringenin (4). *R. meliloti* has also been found to be chemotactic to 7,4'-dihydroxyflavone. Although chromosomal genes in rhizobia are involved in chemotactic responses to carbon substrates (81), plasmid genes *nod* D and *nod*ABC genes carried on *pSym* contribute to the flavonoid induced chemotaxis.

Bacterial growth and metabolism — Low concentrations of flavonoids have been reported to influence growth rate of rhizobia. Isoflavonoids, medicarpin and kievitone from soybean root strongly inhibited growth of *B. japonicum, R. lupini* and two fast growing species of lotus rhizobia, while phaseollin and maakiain had moderately inhibitory effects. However, the growth of *R. trifolii, R. leguminosarum* and *R. meliloti* was not affected by any of these compounds (82). D'Acry Lameta and Jay (83) reported that low concentration of daidzein enhanced the growth of one of a *B. japonicum* strain, while higher concentration had no effect. Luteolin, quercetin and flavone enhanced the growth of *R. meliloti* (2,3). Growth enhancement perhaps does not depend on the induction of *nod* genes as quercetin, which is not an inducer of *nod* genes of *R. meliloti*, stimulated its growth. The growth-stimulating phenomenon by flavonoids released

from plants may favour development of certain bacterial population, thereby playing a critical role in structuring the microbial community around the developing root.

Flavonoids have not only been found to stimulate growth of rhizobia but they are also known to affect their metabolism. In *R. meliloti* naringenin was reported to increase the activities of key enzymes of both the phosphogluconate pathway and citric acid cycle, which are the major pathways of sugar metabolism in fast growing rhizobia while the activities of enzymes of pentose phosphate pathway remained unaffected (3). In *Bradyrhizobium* sp. (*Vigna*), both naringenin and syringaldehyde enhanced nitrogenase activity and activities of enzymes of glycolytic pathway (84). Flavonoids were also reported to affect enzymes of ammonia assimilation in *Bradyrhizobium* sp. (*Vigna*) and *R. meliloti* (85). Flavonoid mediated increase in carbohydrate metabolism and ammonia assimilation under free living conditions is perhaps for supporting growth of rhizobia around roots.

Growth of mycorrhizal fungi — Flavonoids are also known to act as important signals to soil microbes other than rhizobia. Root exudates and extracts from pine (*Pinus sylvestris*) enhanced spore germination of several species of *Suillas*, a mycorrhizal fungi, that form actomycorrhizal associations (86). Promotive effect of root exudates on *in vitro* spore germination/or hyphal growth of vascular arbuscular mycorrhizal (VAM) fungi has been documented (87). Commercially available flavonoids have been shown to promote spore germination of VAM fungus, *Gigaspora margarita* (88). It was not surprising therefore, when tests showed some flavonoids released naturally from alfalfa seeds and roots promoted *in vitro* spore germination of two *Glomus* species that form mycorrhizae on alfalfa (89). Luteolin-7-O-glucoside, quercetin and liquiritigenin also enhanced spore germination significantly but 4,4′-dihydroxy-2′-methoxychalcone had no effect, while formononetin significantly inhibited spore germination (88). Whether alfalfa plants exposed to *Glomus* produce additional flavonoids as they do in the presence of *R. meliloti*, is not known.

Conclusions

Flavonoids exuded by plants regulate activities of soil microorganisms at concentrations as low as nano to micromolar levels. Flavonoids regulated processes have been most thoroughly studied in symbiotic nitrogen fixing rhizobia where they induce transcription of nodulation genes required for synthesis of Nod factors which help in nodule initiation and nodule functioning. The flavonoids also influence chemotaxis, growth and metabolism of rhizobia.

Important effects on other soil microbes are evident in the capacity of some flavonoids in seeds and root exudates to promote spore germination and hyphal growth of mycorrhizal fungi. The amount and identity of flavonoids released from different crop plants offer a basis for molecular, genetic and ecological studies on how these compounds may control rhizosphere biology and soil formation. Since flavonoids are biologically active at such low concentrations, it will be important to determine whether their presence in soil is controlled primarily by their release from living and dead plants, or by their inactivation through biological and chemical processes. A large number of flavonoids present in varying proportions in the rhizosphere has beneficial as well as harmful effects on soil and rhizosphere biology. Quantitative and qualitative manipulations of flavonoids in rhizosphere by biological and/ or chemical means offer an exciting area of research for achieving desired output from soil-microbe-plant interactions.

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