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Flavonoids as plant signals to rhizosphere microbes

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Summary. Certain flavonoids released from plants regulate activities of soil microbes at micromolar and nanomolar concentrations. Processes affected by these compounds include induction of nodulation gene transcription in rhizobial bacteria, promotion of chemotaxis in rhizobia, increase in growth rate of several bacterial species, and enhancement of *Glomus* spore germination and hyphal growth. Data on the amount and identity of flavonoids released from several crop plant species form a new basis for molecular genetic and ecological studies of the rhizosphere.

Key words: *Glomus* – Nodulation – *Rhizobium* – Spore germination

Introduction

The concept of a plant rhizosphere is based on the well-known fact that roots promote microbial growth by releasing significant amounts of carbon into carbon-deficient soil environments (Whipps and Lynch 1986). The fact that some carbon released from roots is in the form of flavonoids was first reported by Lundegardh and Stenlid (1944), but only recently were the structure and quantities of a few of those compounds characterized (D'Arcy-Lameta 1986). With the recent demonstration that certain flavonoids induce transcription of genes in *Rhizobium* at a concentration several orders of magnitude lower than other microbial gene inducers (e.g., Peters et al. 1986), it became obvious that more information is needed on the release of these compounds by plants. The purpose of this report is to summarize recent data on the role of flavonoids in *Rhizobium*-legume interactions and to suggest how that information may benefit mycorrhizal studies. How plant phenolics regulate infection by *Agrobacterium* and parasitic angiosperms is summarized elsewhere, as are broader concepts of molecular communication between plant and microbial pathogens (Dixon and Lamb 1990; Lynn and Chang 1990) and will not be discussed here.

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Flavonoid effects on rhizobia

Flavonoids released from legumes (e.g., those in Fig. 1) affect root nodulation (1) by inducing transcription of rhizobial nodulation (*nod*) genes required for infection, (2) by promoting bacterial movement toward the plant, and (3) by enhancing the growth rate of bacterial cells. Initial reports identified only a few active flavonoids, but detailed studies now show that a broad range of such molecules is released by individual legume species. Exact mechanisms by which plants control the synthesis and/or release of these potent compounds and how various bacterial responses are triggered remain to be defined.

Early observations that host legume factors are required for transcription of some *nod* genes in *Rhizobium* and *Bradyrhizobium* bacteria (e.g., Mulligan and Long 1985) quickly led to the identification of particular flavonoids in different legumes (Firmin et al. 1986; Peters et al. 1986; Redmond et al. 1986; Kosslak et al. 1987). The list of active *nod*-gene-inducing flavonoids released from various host legumes has now become quite long, as other scientists have isolated additional active factors from seed rinses and root exudates (Table 1). The most detailed data on structures of *nod*-inducing flavonoids released naturally into aqueous solution by sterile legumes have been reported for alfalfa (*Medicago sativa* L.) (Peters et al. 1986; Maxwell et al. 1989; Hartwig et al. 1990a) and *Phaseolus vulgaris* L. (Hungria et al. 1991a, b).

Flavonoids control *nod* gene transcription by interacting in some way with products of the regulatory

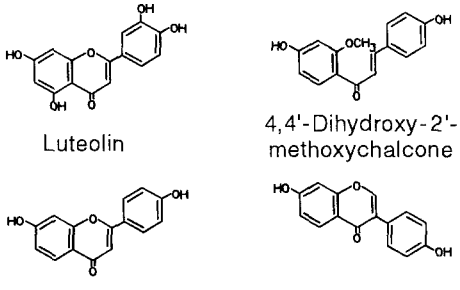

Fig. 1. Examples of flavonoid *nod*-gene inducers from various legumes

Table 1. Flavonoid *nod* inducers from sterile legumes

Source	Compound(s)	Reference
Alfalfa seed extract	Luteolin (3′,4′,5,7-tetrahydroxyflavone)[a]	Peters et al. (1986)
Alfalfa root exudate	4,4′-Dihydroxy-2′-methoxychalcone[a] 4′,7-Dihydroxyflavone[a] 4′,7-Dihydroxyflavanone	Maxwell et al. (1989)
Alfalfa seed rinse	Luteolin Chrysoeriol (3′-methoxyluteolin)	Hartwig et al. (1990)
Pea seed rinse	Eriodictyol (3′,4′,5,7-tetrahydroxyflavanone) Apigenin-7-*O*-glucoside (4′,5,7-trihydroxyflavone-7-*O*-glucoside)	Firmin et al. (1986)
Vetch root exudate	3,3′,5,7-Tetrahydroxy-4′-methoxyflavanone Six other unidentified inducers	Zaat et al. (1989)
White clover seedling extract	4′,7-Dihydroxyflavone[a] Geraldone (4′,7-dihydroxy-3′-methoxyflavone) 4′-Hydroxy-7-methoxyflavone	Redmond et al. (1986)
Soybean seedling and seed extracts	Genistein (4′,5,7-trihydroxyisoflavone) Daidzein (4′,7-dihydroxyisoflavone)[a]	Kosslak et al. (1987) Sadowsky et al. (1988)
Common bean seed rinse	3-*O*-glycosides of anthocyanidins (delphinidin, petunidin, malvidin) 3-*O*-glycosides of flavonols (myricetin, quercetin, kaempferol)	Hungria et al. (1991a)
Common bean root exudate	Genistein-7-*O*-glycoside Eriodictyol Naringenin (4′,5,7-trihydroxyflavanone)	Hungria et al. (1991b)

[a] Structure shown in Fig. 1

nodD gene family (Long 1989). One working hypothesis is that the *nodD* gene product binds DNA and is activated by the proper flavonoid to permit transcription. Although flavonoids are molecular on-off switches that control root nodule formation, numerous classical data indicate that root nodulation is strongly affected also by the number of rhizobial cells present (Vincent 1974). Thus the induction of rhizobial cells is a molecular event, but the formation of an optimum number of nodules is a population phenomenon requiring a large number of induced bacteria. In two cases, flavonoids added to the rhizosphere increased root nodule formation and N_2 fixation in alfalfa under controlled conditions (Kapulnik et al. 1987; Jain et al. 1990). Apparently this response involves many factors because it cannot be demonstrated with all alfalfa cultivars (Y. Kapulnik, personal communication).

The first role described for flavonoids in root nodulation involved their induction of *nod* genes, but subsequent studies showed that they also serve as chemoattractants for rhizobia at nanomolar and micromolar concentrations (Aguilar et al. 1988; Armitage et al. 1988; Caetano-Anolles et al. 1988). Interestingly, mutations in *nodD* eliminate the chemotactic response of *Rhizobium meliloti* to luteolin (Caetano-Anolles et al. 1988). Thus movement of rhizobia toward alfalfa and the induction of genes required for root nodule formation are apparently both affected by the same compound.

Several lines of investigation indicate that plant flavonoids can alter bacterial growth. It has been known for many years that legume seeds release compounds that inhibit rhizobial growth (Thompson 1960), and the flavonol myricetin was identified as one such molecule (Fottrell et al. 1964). Daidzein, a *nod* gene inducing isoflavone from soybean (Kosslak et al. 1987), increased the growth rate of *Bradyrhizobium japonicum* (D'Arcy-Lameta and Jay 1987) but unexplained concentration and strain effects complicated interpretation of that report. In alfalfa, two of the dominant flavonoids released from imbibing seeds, luteolin-7-*O*-glucoside and quercetin-3-*O*-galactoside, enhanced the growth rate of all *R. meliloti* strains tested on a minimal medium, and the response saturated at 1–5 μM (Hartwig et al. 1991). The aglycones luteolin and quercetin, which presumably are produced readily from the glycosides by enzymatic processes in the soil, were responsible for the activity, and experiments showed that the 5,7-dihydroxyflavone portion of the molecules was responsible for the effect. Apparently the growth effect of flavonoids is controlled separately from *nod* gene transcription, because a strain of *R. meliloti* mutated in all *nodD* genes still grew faster with quercetin and luteolin.

Because alfalfa releases structurally different flavonoids from seeds and roots, there may be two ecochemically different zones around a very young seedling. Most flavonoids released from seeds have substitutions on the C-5 position, and nearly two-thirds of that fraction is composed of luteolin and quercetin (3-hydroxyluteolin) derivatives (Hartwig et al. 1990a, 1991). In contrast, the root exudes primarily 5-deoxy flavonoids (Maxwell et al. 1989). Luteolin and quercetin, but not the 5-deoxy flavonoids from roots, enhance the growth rate of *R. meliloti* (Hartwig et al. 1991). There may be special flavonoid effects on *nod*-gene transcription where the root and seed zones overlap, because mixtures of 4,4′-dihydroxy-2′-methoxychalcone from roots and luteolin from seeds have a synergistic effect on *nod*-gene induction (Hartwig et al. 1989). Although the molecular basis of that effect is not clear, these mole-

cules interact differently with various *nodD*-gene products in *R. meliloti* (Hartwig et al. 1990b).

Alfalfa plants apparently can influence total *nod*-gene-inducing activity in the rhizosphere through several mechanisms. For example, a strong link between synthesis and exudation of *nod*-gene inducers from the root (Maxwell and Phillips 1990) suggests the plant may decrease *nod*-inducing activity in the rhizosphere by stopping synthesis of these molecules. Alternatively, the plant might release more of the weak *nod*-gene inducer 4′,7-dihydroxyflavanone and allow that molecule to decrease the strong inducing effects of luteolin through competitive binding to an active site on the *nodD*-gene product (Hartwig et al. 1989). Germinating alfalfa seeds, likewise, can probably alter total *nod* induction in *R. meliloti* because sterilized seeds release glucosidase activity that hydrolyzes the inactive *nod* inducer luteolin-7-*O*-glucoside to the strong *nod* inducer luteolin (Hartwig and Phillips 1991). The importance of the overall process of using plant flavonoids to induce rhizobial *nod* genes is emphasized by the fact that both the alfalfa plant and *R. meliloti* have evolved redundancies in critical steps: the plant releases a variety of flavonoids capable of inducing *nod* genes, while the bacterium has three homologous copies of *nodD* (Honma and Ausubel 1987).

Flavonoids and mycorrhizal fungi

If legumes use flavonoids to control the early stages of their association with symbiotic rhizobia, it is possible that analogous mechanisms influence plant symbioses with mycorrhizal fungi. Thus one can postulate that flavonoids or other small molecules from plants may influence mycorrhizal fungal spore germination, hyphal growth, and/or infection. Positive effects of pine seedling roots on spore germination of the ectomycorrhizal fungus *Hebeloma* were reported in 1980 (Fries and Birraux 1980), and an active factor from pine roots was identified as the diterpenoid abietic acid, a $C_{20}H_{30}O_2$, nonflavonoid molecule (Fries et al. 1987). More recently it was shown that white clover root exudates stimulate hyphal elongation of *Glomus fasciculatus* (Elias and Safir 1987), and Gianinazzi-Pearson et al. (1989) reported that three flavonoids, apigenin, hesperitin and naringenin, promoted spore germination and hyphal growth of *Gigaspora margarita* at concentrations of 0.15–1.5 μM. The fact that vesicular-arbuscular mycorrhizal (VAM) fungi induce isoflavonoid accumulation in infected roots (Morandi et al. 1984) is consistent with normal plant responses to fungal pathogens. Whether flavonoids produced by plants in response to VAM fungal infection influence the development of these fungi in the root remains to be tested. When genetic systems are developed for the study of VAM fungi, it may be possible to ask whether flavonoids affect specific transcriptional events, as they do in rhizobia.

If flavonoids affect VAM fungal development in vivo, then the same molecules may be important factors for optimizing in vitro culture methods for these organisms. Thus we have tested flavonoids that occur naturally in the seed and root zone of alfalfa seedlings for their effects on the in vitro development of *Glomus etunicatum* spores. In preliminary experiments on water agar, quercetin, the dominant aglycone released from alfalfa seeds (Hartwig et al. 1991), markedly promoted spore germination, hyphal growth and hyphal branching, while 4′,7-dihydroxyflavone, the major flavonoid aglycone exuded by young alfalfa roots (Maxwell et al. 1989), primarily enhanced hyphal branching (Fig. 2). Subsequent experiments have shown that responses to flavonoids shown in Fig. 2 saturate at concentrations lower than 10 μM when nutrients are supplied with the flavonoid in the water agar (Tsai and Phillips 1991). Under those conditions, 4′,7-dihydroxyflavone strongly promotes spore germination of *G. etunicatum*. Any roles of quercetin and/or 4′,7-dihydroxyflavone in controlling *G. etunicatum* under field conditions remain to be determined.

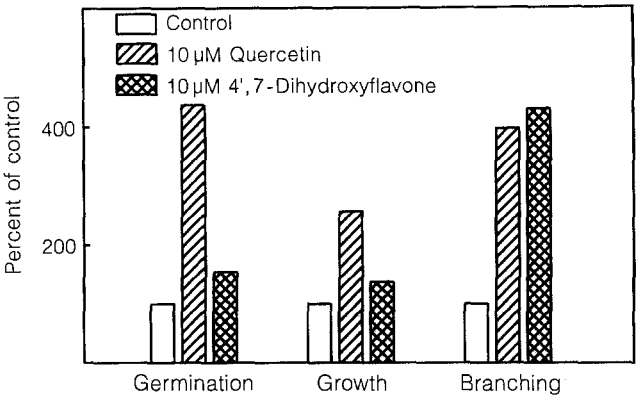

Fig. 2. Effects of the natural alfalfa flavonoids quercetin and 4′,7-dihydroxyflavone on *Glomus etunicatum* spores in vitro. Flavonoids were supplied in water agar at 10 μM and spores were scored 28 days later for germination, hyphal growth and hyphal branching

Flavonoids as control factors in soil biology

Flavonoids may influence more than just symbiotic microbes in the soil through their effects on microbial growth. The positive effect of luteolin and quercetin on bacterial growth rate is not specific for *R. meliloti* but neither is it a general phenomenon affecting all microbes occurring around a young alfalfa seedling (Hartwig et al. 1991). The same compounds promote growth of the benign soil bacterium *Pseudomonas putida* while having no effect on *Bacillus subtilis* or *Agrobacterium tumefaciens*. Treatment with these flavonoids at 10 μm decreased the in vitro growth of *Pythium irregulare* and *P. ultimum*, two fungal pathogens on alfalfa. Thus it may be instructive to examine how flavonoids released naturally from plants influence soil microbial populations. The critical effects of soil microbes on organic matter content and structural properties of soil are well known, and determining whether flavonoids are controlling factors in these processes may benefit agricul-

ture. If, for example, particular flavonoids are identified as factors that promote growth of microbes important for mineral cycling or disease suppression, those compounds might be supplied by growing particular genotypes of crop plants which exude the desired flavonoids from living roots or release them from decaying plant material.

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