Role of Flavonoid and Isoflavonoid Molecules in Symbiotic Functioning and Host-Plant Defence in the Leguminosae

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Abstract Inoculating symbiotic legumes with infective rhizobial symbionts increases the nod-gene-inducing activity of root exudates, and alters the profile of nod gene inducers. The application of *Sinorhizobium meliloti* cells to the roots of alfalfa seedlings specifically causes the release of the aglycone and glycoside forms of the phytoalex in medicarpin, and a formononetin—O-(6″-O-malnylglycoside). Similarly, in the presence of *Rhizobium leguminosarum* biovar *phaseoli* bacteria, root exudates of common bean also contain more of the phytoalexin coumestrol, and its isoflavonoid precursor daidzein than exudates of uninoculated plants. This paper discusses the effects of root-nodule bacteria (hereafter called "rhizobia") on the synthesis and release of flavonoid and isoflavonoid signal compounds, and explores the biological significance of phytoalexin production in legume plant nodulation and defense against pathogens and insect pests.

1 Introduction

Nitrogen, phosphorus, and water are the most limiting factors to increased crop yields in Africa. With the high cost of chemical fertilizers, interest has increased in seeking new approaches to promote N nutrition in crops. In global terms, biological N_2 fixation contributes about 65 % of N used in agriculture today, and therefore provides an

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alternative to chemical fertilizers. N_2 fixation in root nodules of symbiotic legumes represent the major source of N for food production in the traditional cropping systems in Africa. During nodule formation, some legumes release flavonoid and isoflavonoid signal molecules, which act as chemo-attractants to invading root-nodule bacteria or "rhizobia" [1]. Additionally, these phenolic molecules fulfill various other functions, including promotion of bacterial growth [2] and induction of nodulation (*nod*) genes in compatible bacterial strains [3, 4]. Different legumes release different profiles of flavonoids and isoflavonoids from their roots and germinating seeds. Alfalfa grown in the absence of its N_2-fixing symbiont, releases five distinct flavonoids [3, 5, 6] and two betaines [7] that induce transcription of *nod* genes in homologous *Sinorhizobium meliloti.* The common bean plant also releases nine compounds from seeds and roots that cause expression of *nod* genes in its microsymbiont [8, 9], while sterile *Vicia* roots release only two *nod* gene-inducing compounds [10].

The early steps in nodule formation involves a two-way molecular communication between the legume and rhizobial symbiont. Flavonoid and isoflavonoid signal molecules released by leguminous host induce expression of rhizobial *nod* genes followed by a coordinated synthesis of rhizobial signal compounds, such as lipo-chito-oligosaccharide nod factors. It is these nod factors that affect morphological changes in legume root hairs, leading to nodule formation. In compatible interactions, rhizobial signaling to host root hairs results in root hair deformation, root hair branching, root hair curling, cortical cell division, infection thread formation [11], and ultimately nodule development. It is in the mature nodules that N_2 fixation occurs through the activity of the enzyme nitrogenase.

2 Microsymbiont-Induced Biosynthesis of Flavonoid and Isoflavonoid Compounds in Legumes

Studies with white clover [12] and later *Vicia sativa* subsp.*nigra* [13] were the first to demonstrate that inoculating host legume with infective rhizobial strains increased *nod* gene-inducing activity. Because that finding implied increased production of new and/or existing compounds, Recourt et al. [10] found from analysis of root exudates that, with *Rhizobium* inoculation, *Vicia* released 8 new additional nod gene-inducing compounds. However, there was no evidence that the inoculated roots released isoflavonoid phytoalexins [10] even though most of the 5-deoxy flavonoids produced by inoculated seedings could easily be metabolized to medicarpin, a common phytoalexin in *Vicia faba* [14].

Like *Vicia,* alfalfa root exudates also showed increased *nod* gene-induction following seedling inoculation with *Rhizobium meliloti* [15]. Similarly, root exudates of the common bean exhibited greater nod gene-inducing activity when plant roots were inoculated with *Rhizobium leguminosarum bv phaseoli* compared to uninoculated controls [16]. HPLC analyses followed by NMR and MS studies revealed that, unlike *Vicia*, those two symbioses exuded isoflavonoid phytoalexins in the presence of rhizobial symbionts. *Rhizobium meliloti* elicited the exudation of medicarpin, medicarpin-

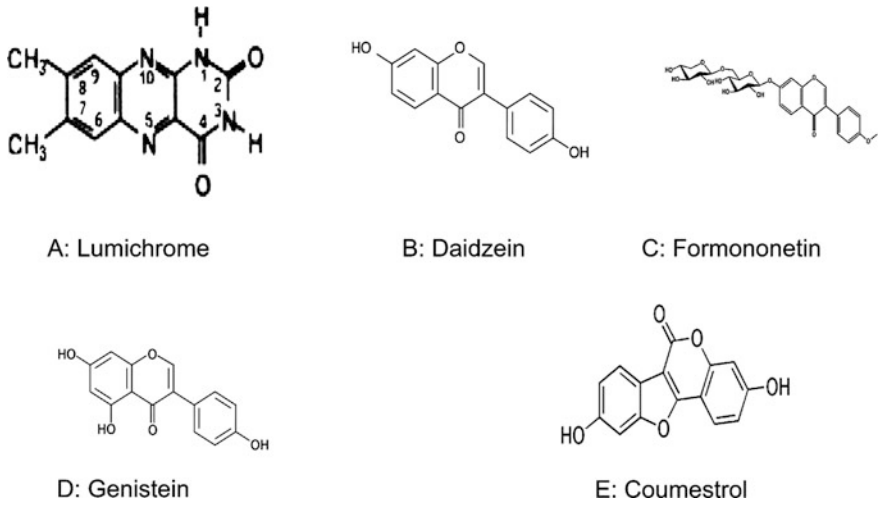
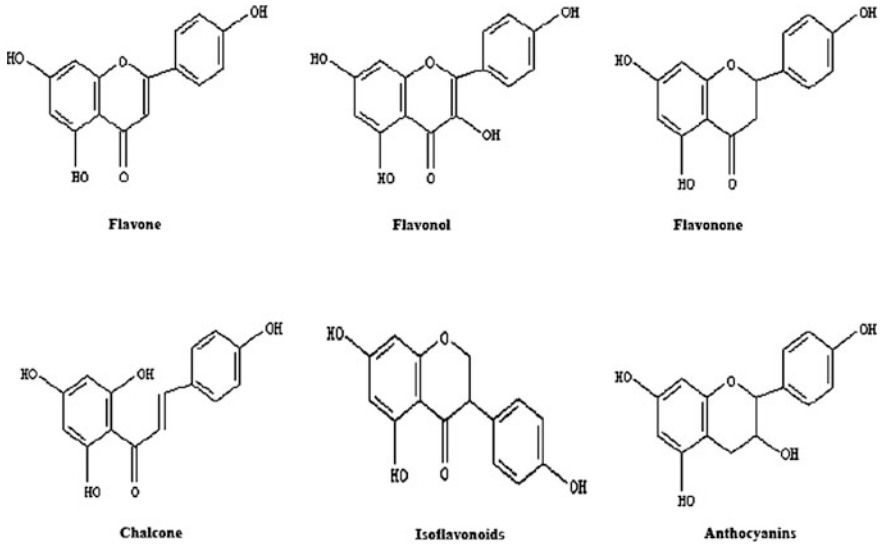


Fig. 1 Flavonoid compounds

3-Oglycoside, and formononetin-7-O-(6″-O-malonylglycoside) from the roots of alfalfa plants. In addition to genistein, eriodictyol, and naringenin which are normally present in root exudates of sterile-grown bean plants [9]; the phytoalexin coumestrol and its precursor daidzein (Fig. 1a) were released by roots of bean in response to *Rhizobium* inoculation [16]. Tests for biological activity showed that the two compounds were active *nod* gene inducers. Thus, coumestrol can function both as a nod gene inducer in bean rhizobia and as a phytoalexin for host plant defense.



Other studies have shown that exposing soybean plants to infective strains of *Bradyrhizobium japonicum* led to the exudation of glyceollin, a major phytoalexin of soybean [17]. However, the amount of glyceollin released in the presence of *Bradyrhizobium japonicum* was less compared to levels exuded in the presence of *Phytophthora* pathogen [17]. With *Sinorhizobium fredii* (another soybean symbiont), however, the level of glyceollin exudation was comparable to that caused by pathogenic *Phytophthora* [17]. Those studies [15–17] clearly indicate that both pathogens and rhizobial symbionts can elicit phytoalexin exudation in N_2-fixing legumes. Even symbiotic fungi have been shown to trigger the formation of isoflavonoid phytoalexins in their host plants. In one study, analysis of soybean root extracts revealed increased tissue concentrations of glyceollin, coumestrol, and its precursor daidzein following *Glomus* infection of roots [18]. In alfalfa, *Glomus* infection resulted in accumulation of only formononetin in the roots [19], suggesting that phytoalexins are formed in both alfalfa and bean in response to fungal symbionts. These findings demonstrate that isoflavonoid phytoalexin production is caused by both pathogenic and symbiotic microbes, so to view these molecules simply as occurring in diseased tissue is misleading.

In contrast, some studies have found no accumulation of phytoalexins with fungal or rhizobial symbionts. Soybean cv. Maple Arrow, for example, showed no accumulation of glyceollin in response to *Glomus mosseae,* though with *Rhizoctonia solani* pathogen, glyceollin accumulation was very high [20]. The observed lack of phytoalexin accumulation in response to mycorrhizal fungus is comparable to *Bradyrhizobium japonicum* infection of soybean roots without inducing phytoalexin in accumulation [21]. However, with loss of symbiotic ability, rhizobial strains can be perceived as pathogens by host plants. For example, infection of soybean root by fix mutants of *Bradyrhizobium japonicum* led to rapid development of necrosis and instant phytoalexin accumulation [21], symptoms reminiscent of pathogen invasion.

Apart from these inconsistencies in overall experimental observations, similarities exist between rhizobial and fungal symbioses, especially with respect to host plant response to symbionts. *Sinorhizobium* inoculation of alfalfa seedlings stimulates the formation and release of conjugated formononetin into root exudates [15]. With mycorrhizal infection, the aglycone form of the same molecule is also found in alfalfa root extracts [19] and possibly exudates, suggesting that host plant recognition of symbiont, be it a fungus or rhizobium, is by the same mechanism. In that regard, mycorrhizal roots have been found to produce proteins which are immunologically related to nodulins in N_2-fixing nodules [22]. Another study has also shown that both pathogens and symbionts of soybean identify their hosts by recognizing the same chemical signals [23].

3 Phytoalexin Effects on Rhizobial Symbionts

Phytoalexin accumulation to higher concentrations in host plant tissue can have detrimental effects, especially where micro-symbiont cells are in contact with such metabolites. An early study by Cruickshank [24] showed that pisatin, (a classical

Table 1 Legume flavonoids and isoflavonoids involved in nod gene expression and N_2 fixation

Legume species	[1]Nod gene-inducing compounds	[2]Amount of N-fixed (kg. N ha^{-1})
Alfalfa	4,4′-dihydroxy-2′-methoxychalcone[a]	2–208[a]
	4′-7-Dihydroxyflavone liquiritigenin[a]	
Cowpea	Daidezein[b]	24–201[b]
	Genistein[b]	
	Coumestrol[b]	
Common bean	Genistein-3-O-glucoside[c]	0–165[c]
	Eridictyol[c]	
	Naringenin[c]	
	Daidzein[c]	
	Genestein[c]	
	Coumestrol[c]	
Soybean	Isoliquiritigenin[d]	26–197[d]
	Genestein[d]	
	Genestein-7-O-glucoside[d]	
	Coumestrol[d]	
	Daidzein[d]	
	Fomononetin[d]	
	Biochanin A[d]	
Bambara groundnut	Daidzein[e]	40–62[e]
	Genestein[e]	
	Coumestrol[e]	
Sesbania	Liquitigenin[f]	505–581[f]

Functional role [a] Ref. [6], [b] Ref. [72], [c] Ref. [73], [d] Ref. [29], [e] Ref. [74], [f] Ref. [75]
N-fixed [a] Ref. [76], [b] Ref. [70], [c] Ref. [77], [d] Ref. [78], [e] Ref. [70], [f] Ref. [70]

phytoalexin from pea and vetch) significantly inhibited growth of rhizobia compared with other rhizosphere microorganisms. Interestingly, *Rhizobium leguminosarum* biovar *viciae*, the microsymbiont of pea and vetch, exhibited a much broader range of pisatin tolerance relative to other rhizobial species. A survey on the inhibitory effects of selected isoflavonoid phytoalexins revealed three levels of sensitivity: pisatin, coumestrol, formononetin, vestitol, biochanin A, genistein, and rotenone were inhibitory; phaseollin, and maackiain, moderately inhibitory; while medicarpin and kievitone were strongly inhibitory [25]. In that study, kievitone and medicarpin, the latter a common photoalexin of agriculturally important legumes, such as *Vigna unguiculata, Cicer arietinum, Canavalia ensiformis, Vicia faba, Medicago, Melilotus*, and *Trifolium* spp., showed considerably marked inhibition of growth in slow-growing root-nodule bacteria compared to fast-growing strains (Table 1). Phaseollin also exhibited significant inhibition of growth in bradyrhizobia but had little effect on fast-growing species.

While the concentrations used in in-vitro studies [24, 25] probably surpass those encountered in plant tissues, the data obtained nevertheless provide a useful indication of the spectrum of symbiont sensitivity to isoflavonoids. Classical phytoalexins produced and released in purely symbiotic systems (Table 2), include medicarpin from alfalfa-*Rhizobium meliloti* symbiosis [15], coumestrol from bean-

Table 2 Mechanisms of bioprotection in plants by symbiotic rhizobia

Interaction	Chemical molecule	Reference
Alfalfa-*Rhizobium melitoti*	Medicarpin	[15]
Bean-*Rhizobium leg.* bv. *viciae*	Coumerstrol	[16]
Soybean-*Bradyrhizobium japonicum*	Glyceolin	[17]
Groundnut-*Bradyrhizobium*	Stilbenes	[79]
Chickpea-*Rhizobium*	Formononetin and biochanin A	[55]
Rice-*Rhizobium leguminosarum bv. phaseoli*	Gallic, tannic, ferulic, and cinnamic acids.	[43]
Clover-*Rhizobium*	Biochanin A–7-O-glucoside–malonate	[80]

Rhizobium leguminosarium biovar *phaseoli* interaction [15], coumestrol from bean-*Rhizobium leguminosarum* biovar *phaseoli* interaction [16], and glyceollin from soybean-*Bradyrhizobium japonicum* association [17]. Although the in-vitro sensitivity of *Rhizobium meliloti* to medicarpin, the major phytoalexin of alfalfa, is low, it is difficult to imagine how the bacterial symbiont copes with increasing metabolite concentration in tissues and in the rhizosphere. Initial experiments revealed that at 150 micromolar concentration, glyceollin inhibited growth of *Bradyrhizobium japonicum* [26], suggesting that the microsymbiont must have a mechanism for overcoming glyceollin toxicity in soybean tissue and/or rhizosphere.

Various mechanisms have been suggested for phytoalexin tolerance in pathogens and symbionts. Phytoalexin detoxification is one such mechanism by which microbial symbionts and pathogens overcome plant defence response. The phytoalexins medicarpin and maackiain, which are produced by a number of legumes, can be degraded by *Nectria haematococca* and *Ascochyta rabiei*, (two common fungal pathogens of chickpea and other legumes) via NADPH-dependent reductase conversion of the molecule to less toxic isoflavan, isoflavanone, or 6a-hydroxyptercocarpan (see Van Etten et al. [27]). Whether *Rhizobium meliloti* employs such a mechanism to detoxify medicarpin and other phytoalexins, remains to be seen Table 3.

The bean pathogen, *Fusarium solani* f. sp. *Phaseoli,* can also detoxify four major bean phytoalexins, namely kievitone, phaseollin, phaseollidin, and phaseollinisoflavan; kievitone detoxification occurs through kievitone hydratase-catalyzed hydration of the isopentenyl side chain to yield the less toxic kievitone hydrate [27]. There is evidence that *Rhizobium* and *Bradyrhizobium* strains modify various flavonoid molecules within their vicinity [28], suggesting that bacterial and fungal symbionts probably overcome isoflavonoid phytoalexin toxicity in tissues and soil through biotransformation of these compounds.

Besides chemical modification, bacterial tolerance of phytoalexins may be flavonoid-induced. Growing cells of a pathogen or symbiont in low-level concentrations of a phytoalexin can confer resistance to the microbe against the toxic effects of phytoalexins. It has been shown recently that this induction of resistance against phytoalexins may be effected by low concentrations of a second,

Table 3 Isoflavonoid compounds from legume roots with potential for use as defense molecule against soil borne pests (insect larvae and pathogens) in cropping systems

Legume species	Isoflavonoid	Defense role	Reference
Lotus pedunculatus	Vestitol	Insect deterrent	[81]
Glycine max	Glyceollin	Insect deterrent phytoalexin	[71]
Vigna unguiculata	Medicarpin	Insect deterrent phytoalexin	[71]
Phaseolus vulgaris	Phaseolin	Insect deterrent phytoalexin	[71]
Phaseolus linatus	Coumestrol	Nematicide	[71, 82]
Cajanus cajan	Cajanin	Insect deterrent phytoalexin	[71]
Lonchocarpus nicou	Rotenone	Insecticide phytoalexin	[83]
Derris malaccensis	Rotenone Deguelin Sumatrol taxicarol	Insecticide phytoalexin	[83]
Mundulea serica	Munduserone	Insecticide phytoalexin	[83]
Pachyrrhizus erosus	Pachrrhizone	Insecticide phytoalexin	[83]
Neoratanenia pseudopachyrrhiza	Dolineone	Insecticide phytoalexin	[83]

structurally-related molecule other than the phytoalexin itself. In a classical study by Parniske et al. [26], it was demonstrated that preculturing *Bradyrhizobium japonicum* with 10 μmM concentration of genistein or daidzein induced resistance in the bacterium against the phytoalexin glyceollin. In that study, cell viability tests involving bacterial growth in geneistein-free medium followed by transfer to 300 μM glyceollin showed a strong bactericidal effect, while rhizobia precultured with genistein were unaffected. Kape et al. [29] have also found that isoliquiritigenin (2′,4′,4-trihydroxychalcone) is both a strong nod gene inducer and glyceollin resistance inducer. This confirms the role of flavonoids in phytoalexin resistance induction. While these findings may be the first for bacterial symbionts, induced phytoalexin resistance is not new in fungal pathogens. Denny van Etten [30, 31] have reported and induced resistance to pisatin in *Nectria haematococca.*

Although experimental results show that isoflavonoids can induce resistance in soybean microsymbionts against glyceollin phytoalexins [26], the mechanism by which this resistance is induced is still not properly understood. It is, however, clear that, unlike rhizobial *nod* gene induction which requires flavonoid interaction with *nod*D protein, induction of glyceollin resistance in *Bradyrhizobium* does not involve the common *nod* genes. This was evidenced by the successful induction of glyceollin resistance in a *nod*D_1D_2YABC deletion mutant [26]. Equally unknown is the mechanism by which soybean rhizobia and pathogens overcome the inhibitory effects of glyceollin. Even the mode of action of phytoalexins on pathogens and/or symbionts remains undefined. In the case of the glyceollins,

however, the lethal effect of these phytoalexins on pathogens and symbionts is exerted via inhibition of plasma membrane and tonoplast H^+-transporting ATPases [32], and/or NADH-ubiquinone-oxidoreductase [33].

4 Nodulation Significance of Phytoalexins From Legumes

The release of phytoalexin compounds by legume roots, whether in response to pathogen invasion, symbiont infection, or both, is likely to lead to higher concentrations of phytoalexins in the rhizosphere. Root exudation of phytoalexins, such as medicarpin, coumestrol, and glyceollin is known to occur from challenge by symbionts [15–17] and pathogens [26]. This suggests that the rhizosphere of nodulating legumes must be the site of continuous accumulation of phytoalexins triggered by invading rhizobia, root-borne pathogens, and various environmental stimuli. Also, because phytoalexin exudation is a localised response in roots, rhizosphere soil associated with symbiotic field legumes is likely to consist of spatial, and temporal phytoalexin concentration gradients. The accumulation of *nod*-gene inducing isoflavonoid phytoalexins in the rhizosphere is likely to increase the chance of enhancing nodulation while warding off pathogens. In fact, differences in legume nodulation have been shown to relate to limitation in the synthesis and release of *nod* gene-inducing flavonoids [34, 35].

A number of studies [36] have shown that soybean plants release the isoflavones daidzein and genistein into root exudates and these induce nod genes in *Bradyrhizobium japonicum*. Coumestrol is another phytoalexin from soybean which moderately induces *nod* genes in the host's microsymbiont [37]. Thus, the nodulation potential of soybean depends largely on adequate availability of daidzein, genistein, and coumestrol in the rhizosphere to transcribe *nod* genes in *Bradyrhizobium japonicum*. Besides *nod* gene induction, daidzein, and coumestrol released into the rhizosphere also promote growth of the soybean bacterial symbiont [38]. The growth-promoting effect of these isoflavonoids on bradyrhizobial populations in the rhizosphere increases the chance for enhanced nodulation and N_2 fixation in soybean from increased rhizobial numbers. Furthermore, in soybean, daidzein, genistein, and their glycosyl conjugates act in concert against pathogen invasion of this legume [39], resulting in healthy plant growth for better nodulation.

Phytoalexins released by legume roots play other roles essential for nodule formation. As indicated before, the isoflavonoids daidzein and genistein can each independently induce glyceollin resistance in *Bradyrhizobium japonicum* [26], thus permitting selective development of bacterial strain population needed for host plant nodulation. This means that with soybean release of daidzein and genistein into the rhizosphere, bradyrhizobia are induced to develop resistance to glyceollin exuded by legume to ward-off pathogens. Nutritionally, this offers a competitive advantage to microsymbiont, and enhances its ability to survive in the rhizosphere of phytoalexin-producing host roots. Biochemically, daidzein and genistein are important precursors for glyceollin formation [40], indicating that the synthesis and release of glyceollin

would depend on the pool size of isoflavones in tissues as well as their rates of exudation from roots. The ecological significance of induced glyceollin resistance is not only to increase the survival of the microsymbiont in the rhizosphere which is constantly challenged by microsymbionts, pathogens, and environmental stimuli, but also to provide bacteria with physiological plasticity for adaptation to changing phytoalexin concentrations within the rhizosphere.

A recent study has shown that alfalfa growing in iron-deficient medium releases the phytoalexin 2-(3′5′-dihydroxyphenyl)-5,6-dihydroxybenzofuran, which solubilises iron from insoluble ferric compounds for the plant, while controlling *Fusarium oxysporum* f. sp. *phaseoli*, a root pathogen of alfalfa [41]. It is, therefore, likely that many legumes growing in poor soils, use compounds such as isoflavonoids to enhance nutrient uptake and control pathogens.

Coumestrol phytoalexin inhibits growth of pathogens, and acts as *nod*-gene inducer in strains of *Rhizobium leguminosarium* bv *phaseoli*, the causal organism of nodulation in the common bean. The molecule also moderately transcribes *nod*-genes in *Bradyrhizobium japonicum*, the microsymbiont of soybean. In soybean, isoliquiritigenin is both a nod gene inducer and glyceollin resistance inducer [29]. Breeding for increased levels of such compounds in legumes for enhanced nodulation and improved disease control could be useful for agriculture. Alternatively, increased nodulation and pathogen control can be achieved directly and together by applying isoflavonoid phytoalexin inducer compounds to field legumes.

5 Symbiotic Rhizobia as Biopesticides Against Plant Pathogens

Novel findings from field and glasshouse studies have shown that inoculating legumes and non-legume plants with N_2-fixing rhizobia can provide protection against pathogens. In common bean the severity of *Fusarium* root rot was reduced by inoculating it with rhizobia. Similarly, living and heat-killed bacterial cells of *Rhizobium leguminosarum* provided total protection against pathogen infection of lentil plants [42]. That study further revealed that the culture filtrate and the killed bacterial cells contained signals able to induce plant resistance. However, those signals were suppressed once *Rhizobium* was in contact with the plant. Mishra et al. [43] showed that in *Rhizobium*-inoculated rice plants, synthesis of phenolic compounds was consistently more enhanced than in the control, and maximum accumulation of phenolic compounds was observed in plants co-inoculated with *Rhizobium leguminosarum* bv. *phaseoli* and *Rhizoctonia solani*.

Phenolic acids mediate induced systemic resistance and provide bioprotection to plants during pathogenic stresses. In a related study, Khaosaad et al. [44] showed that arbuscular mycorrhizal fungi (AMF) root colonization provides a bioprotectional effect against a broad range of soil-borne fungal pathogens, including take all disease caused by *Gaeumannomyces gramminis var. tritici*. Infection by AMF also enhances shoot and root growth in wheat plants infected with *Gaeumannomyces gramminis*

var. tritici compared to non AMF colonized diseased plants. Similarly co-inoculation of *Rhizobium leguminosarum* and arbuscular aycorrhizal fungi conferred resistance to *Bothytis fabae* in *Vicia faba* as a result of elevated Na-uptake and phenolics concentration in the bean plant [45].

Rhizobia are major biocontrol agents in natural and agricultural ecosystems. There is evidence that a strain of *Bradyrhizobium japonicum* can cause up to 75 % decrease in sporulation of *Phytophthora megasperma*, 65 % in *Pythium ultimum*, 47 % in *Fusarium oxysporum*, and 35 % in *Ascochyta imperfecta* [46]. Antoun et al. [47] identified 49 strains of *Sinorhizobium meliloti* that inhibited growth of *F. oxysporum* by up to 50 %. Rhizobia isolated from root nodules of *Acacia pulchella* similarly decreased the survival of the zoospores of *Phytophthora cinnamoni* in vitro [48], thus potentially providing bioprotection for the host plant.

Field and glasshouse studies show that inoculating plants with rhizobia can be a cheap and effective method of controlling soil-borne pathogens in cropping systems. For example, inoculating soybean and common bean plants with their respective microsymbionts significantly decreased the severity of *Phytophthora* and *Fusarium* root rot in these species [46, 49]. As with most parasitic interactions, the level of root rot decreased with increasing rhizobial numbers in soil [46]. Whether applied as seed dressing or soil drench, different rhizobial strains successfully protected field-grown soybean, mungbean, sunflower, and okra plants from infection by the root-borne pathogens *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Fusarium* species [34]. Although Tu [46] has suggested that rhizobia achieve this bioprotection by parasitizing the hyphal tips of the fungal pathogens and decreasing contact with the host plant cells, other mechanisms may exist. For example, the elicitation of isoflavonoid phytoalexins by rhizobial cells [15, 16] and/or by their *nod* factors [50] can indirectly control pathogens in legumes. However, it is still unclear whether the same protection can be achieved in non-legume hosts such as the cereals and vegetables in mixed cropping systems.

Similar observations of biocontrol of plant pathogens have been reported for mycorrhizae, the second most important mutualism after the rhizobial symbiosis. Following infection, the AM fungus *Glomus mosseae* is claimed to confer bioprotection against *Phytophthora parasitica* in roots of tomato plants [51]. This was shown by the presence of pathogenesis-related proteins in both mycorrhizal and non-mycorrhizal roots, suggesting that the tomato plant probably acquired both localized and systemic resistance against the pathogen. This observation parallels the finding that inoculating legumes with infective rhizobial cells [15, 16], or with their *nod* factors [50], induces the synthesis, and release of isoflavonoid phytoalexins that confer bioprotection to the plant. Interestingly, some rhizobia are themselves protected from the antimicrobial effects of induced phytoalexins by their nod gene inducers [26, 29]. Thus, pathogens are kept under control as rhizobia infect their host-plant roots. Although no specific studies have been done, it is possible that root hair infection by rhizobial bacteria also induces resistance against various pathogens of the host plant, as observed with *G. mosseae* [51]. Hopefully, future studies will provide direct evidence for this claim. Biological control of fungal pathogens can therefore be achieved through a range of

mechanisms, including mycoparasitism, production of phytoalexins, the induction of host plant defense proteins and peptides, as well as competition for nutrients [52]. Tu [46] suggested parasitisation hyphal tips by rhizobia. Purified *nod* factors from rhizobia induced the biosythesis and increased exudation of phytoalexins (daidzein, genistein, and coumestrol) from soybean roots [53]. Cyclic β-glucans produced by bradyrhizobia elicit glyceollin, a phytoalexin that controls pathogens in soybean [53]. Inoculating alfalfa with *Sinorhizobium* induced the release of medicarpin, a classical phytoalexin that control fungal pathogens of the host plant [15]. Similarly inoculating *Phaseolus vulgaris* bean with *Rhizobium leguminosarum bv phaseoli* elicited the exudation of coumestrol, daidzein, and genistein, isoflavonoid phytoalexins that control many fungal and bacterial pathogens of beans and other legumes. *Bradyrhizobium japonicum* secretes rhizobitoxine which inhibits charcoal rot fungus, *Macrophomina phaseolina* [54].

The pre-treatment of chickpea seedlings with *Rhizobium* isolates before challenging them with *Fusarium spp.* significantly increased levels of total phenolics and the levels of constitutive isoflavonoids such as formononetin and biochanin A [55]. It has been suggested that pathogen invasion there is increased the mRNA of the defense gene encoding phenylalanine ammonia lyase (PAL) and leading to the biosythesis of high levels of secondary phenolic metabolites.

Limited data are currently available on flavonoids as signaling compounds for fungal pathogens of legumes. Information about possible effects of flavonoids in root exudates on root infecting/colonizing fungi of non-legume plants remains scanty. Ruan et al. [56] have, however, shown that certain flavonoids, including isoflavonoid phytoalexins, stimulate spore germination of *Fusarium solani formae specia-les* pathogenic on pea and bean. Thus flavonoids in legume root exudates may be perceived as signals in a number of plant–microbe interactions [56]. For example, Steinkellner et al. [57] have reported that the flavonoid compounds, myricetin and luteolin, exhibited a low stimulating activity on microconidia germination of *Fusarium oxysporum* f. sp. *lycopersici*.

6 Mechanisms for *Rhizobium* Control of Plant Diseases

Many studies have provided both glasshouse and field evidence for biocontrol of plant pathogens by symbiotic rhizobia. These include *Phytophthora* root rot control by *B. japonicum* [46, 58], *Fusarium* root rot by *Sinorhizobium melitoti* [59], *Fusarium* root rot, bean bacterial wilt, and *Fusarium* wilt control by *Rhizobium leguminosarum* ([42, 49, 60, 61]; see Table 1). However, except for studies by Tu [46, 58], there are no data from microscopy to support rhizobial entry and intercellular/extracellular localization within the host plant cells. Some evidence for rhizobial invasion of host plant cells, especially where the test plants were non-legumes, has been provided by Matiru and Dakora [62]. However, a huge gap still exists in our understanding of possible mechanisms inducing bioprotection with rhizobial inoculation of crop plants.

While it is understood that rhizobial invasion of homologous legume hosts can trigger de novo synthesis of isoflavonoids and/or enhance the production and release of constitutively-present phytoanticipins [63], the same cannot be said of non-legume plants such as sunflower or okra [54]. Even with symbiotic legumes there is evidence of non-rhizobial bacteria present in root nodules. In earlier studies, [64] showed the presence of many non-nodulating agrobacteria-like strains in root nodules of cowpea. [65] also found many non-nodule forming bacteria that cooccupied root nodules of peanut with legitimate peanut rhizobia. Many studies have since shown that cohabitation of legume root nodules by authentic N_2-fixing rhizobia and agrobacteria is a common phenomenon in the nodulation world. In fact, we have recently isolated over 700 bacteria from cowpea nodules and many of them have been found to be unable to incite nodulation in both cowpeas and siratro (F. Pule-Meulenburg and F. D. Dakora, unpublished data). The question that then arises is; could rhizobial protection of host plants at least in the case of homologous legumes be caused by induction of phenylpropanoid pathway by illegal bacterial coinhabitant of legume root nodules? The recent report of large numbers of non-rhizobial bacteria isolated from nodules of symbiotic legumes suggest that the so-called bioprotection by rhizobia actually comes from opportunistic rather than the direct effect of nodule forming rhizobia. New studies are needed to answer these questions.

Plant natural products derived from phenylalanine and the phenylpropanoid pathways are impressive in their chemical diversity and are the result of plant evolution, which has selected for the acquisition of large repertoires of pigments, structural, and defensive compounds, all derived from a phenylpropanoid backbone via plant-specific phenylpropanoid pathway [66]. The phenylpropanoid pathway is known to be easily induced by both biotic and abiotic factors [67]. Petunia flavonoids such as kaemferol and its glycosides accumulate with pollen tube development on stigmas [68]. While this could suggest induction of the phenlpropanoid pathway by penentration of the infection thread down legume root hair, no data currently exist on flavonoid formation in response to rhizobial entry into host plant cells. Assuming this was to happen, it would mean that even with "crack entry" by rhizobia [69] internal invasion of host plant cells could trigger the phenylpropanoid pathway leading to the synthesis and/or release of isoflavones that unintetendedly serve in host plant defense. But for the absence of microscopy data on rhizobial localization in root cells of test plants a similar mechanism could be advanced for possible accumulation of flavonoids with rhizobial application as biopesticide to field crops. There is evidence that root tips incur injury during growth in difficult highly compact soils [67]. Such a wounding can also induce the phenylpropanoid pathway leading to tissue accumulation of newly formed isoflavonoids and increase in phytoanticipins for plant defense [67]. A combination of these factors with rhizobial application could provide significant defense against pathogens because of the high levels of host plant phenylpropanoid compounds.

7 Conclusion

Research has shown that in addition to fixing atmospheric nitrogen, rhizobia can have not only a positive influence on plant growth but can also help in its protection. Research on the use of rhizobia as a protectant against plant diseases is scanty and mechanisms for bioprotection are not yet well understood. Future research should reveal these mechanisms for bioprotection by symbiotic rhizobia.

References

1. Caetano-Annoles G, Christ-Estes D, Bauer WD (1988) Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. J Bacteriol 170:3164–3169
2. Harwig UA, Joseph CM, Phillips DA (1991) Flavonoids released naturally from alfalfa seeds enhance growth rate of *Rhizobium meliloti*. Plant Physiol 95:797–803
3. Peters NK, Frost JW, Long SR (1986) A plant flavone.luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science 233:977–980
4. Redmond JW, Batley M, Djordjevic MA, Innes RW, Kuempel PL, Rolfe BG (1986) Flavones induce expression of nodulation genes in Rhizobium. Nature 323:632–635
5. Harwig UA, Maxwell CA, Joseph CM, Phillips DA (1990) Chrysoeriol and luteolin released from alfalfa seeds induce nod genes in *Rhizobium meliloti*. Plant Physiol 92:116–122
6. Maxwell CA, Hartwig UA, Joseph CM, Phillips DA (1989) Achalcone and two related flavonoids released from alfalfa roots induce nod genes of *Rhizobium meliloti*. Plant Physiol 91:842–847
7. Phillips DA, Joseph CM, Maxwell CA (1992) Trigonelline and stachydrine released from alfalfa seeds activate NodD2 protein in *Rhizobium meliloti*. Plant Physiol 99:1526–1531
8. Hungria M, Joseph CM, Phillips DA (1991) Anthocyanidins and flavonols, major nod-gene inducers from seeds of a black-seeded common bean. Plant Physiol 97:751–758
9. Hungria M, Joseph CM, Phillips DA (1991) Rhizobium nod-gene inducers exuded naturally from roots of common bean (*Phaseolus vulgaris* L.). Plant Physiol 97:759–764
10. Recourt K, Schripsema J, Kinje JW, Van Brussel AAN, Lugtenberg BJJ (1991) Inoculation of *Vicia sativa* subsp. *nigra* roots with *Rhizobium leguminosarum* biovar viciae results in release of nod gene activating tlavanones and chalcones. Plant Mol Biol 16:841–852
11. Fisher RF, Long SR (1993) *Rhizobium*—plant signal exchange. Nature 357:655–659
12. Rolfe BG, Batley M, Redmond JW, Richardson AE, Simpson RJ, Bassam B, Sargent CL, Weinman JI, Djordjevic MA, Dasso FB (1988) Phenolic compounds secreted by legumes. In: Bothe H, de Bruijn FJ, Newton WE (eds) Nitrogen fixation: hundred years after. Gustav Fischer, Stuttgart, p 405
13. Van Brussel AAN, Recourt K, Pees E, Spaink H, Tak T, Wijffelman CA, Kijne JW, Lugtenberg BJ (1990) A biovar-specific signal of *Rhizobium leguminosarum* bv. viciae induces increased nodulation gene-inducing activity in root exudate of *Vicia sativa* subsp. *nigra*. J Bacteriol 172:5394–5401
14. Hargreaves JA, Mansfield JW, Coxon DT (1976) Identification of medicarpin as a phytoalexin in the broad bean plant (*Vicia faba* L.). Nature 262:318–319
15. Dakora FD, Joseph CM, Phillips DA (1993) Alfalfa (*Midicago sativum* L.) root exudates contain isoflavonoids in the presence of *Rhizobium meliloti*. Plant Physiol 101:819–824
16. Dakora FD, Joseph CM, Phillips DA (1993) Common bean root exudates contain elevated levels of diadzein and coumestrol in response to *Rhizobium* inoculation. Mol Plant Microbe Interact 6:665–668
17. Schmidt PE, Parniske M, Werner D (1992) Production of the phytoalexin glyceollin I by soyabean roots in response to symbiotic and pathogenic infection. Bot Acta 105:18–25

18. Morandi D, Bailey J, Gianinazzi-Pearson V (1984) Isoflavonoid accumulation in soyabean roots infected with vesicular-arbuscular fungi. Physiol Plant Pathol 24:357–364
19. VolPin H, Elking Y, Okon Y, Kapulnik Y (1994) A vesicular-arbuscular mycorrhizal fungus (*Glomus intraradix*) induces a defence response in alfalfa roots. Plant Physiol 104:683–689
20. Wyss P, Boller T, Wiemken A (1991) Phytoalexin response is elicited by a pathogen (*Rhizoctonia solani*) but not by a mycorrhizal fungus (*Glomus mosseae*) in soyabean roots. Experientia 47:395–399
21. Werner D, Mellor RB, Hahn MG, Grisebach H (1985) Soyabean root response to symbiotic infection. Glyceollin I accumulation in an ineffective type of soyabean nodules with an early loss of the peribacteroid membrane. Z Narurforsch 40:179–181
22. Wyss P, Mellor RB, Wiemken A (1990) Vesicular-arbuscular mycorrhizas of wild-type soyabean and non-nodulating mutants with *Glomus mosseae* contain symbiosis-specific polypeptides (mycorrizins). Immunologically cross-reactive with nodulins. Planta 182: 22–26
23. Morris PF, Ward EWB (1992) Chemoattraction of zoospores of the soyabean pathogen, *Phytophthora sojae*, by isoflavones. Physiol Mol Plant Pathol 40:17–22
24. Cruickshank LAM (1962) Studies on phytoalexins. IV. The antimicrobial spectrum of pisatin. Aust J Biol Sci 15:147–159
25. Pankhurst CE, Biggs DR (1980) Sensitivity of *Rhizobium* to selected isoflavonoids. Can J Microbiol 26:542–545
26. Parsnike M, Ahlborn B, Werner D (1991) Isoflavonoid-inducible resistance to phytoalexin glyceollin in soyabean rhizobia. J Bacteriol 173:3432–3439
27. Vanetten HD, Mathews DE, Mathews PS (1989) Phytoalexin detoxification: importance for pathogenicity and practical implications. Ann Rev Phytopathol 27:143–164
28. Rao JR, Sharma ND, Hamilton JTG, Boyd DR, Cooper IE (1991) Biotransformation of the pentahydroxy flavone quercetin by *Rhizobium loti* and *Bradyrhizobium* strains (lotus). Appl Environ Microbiol 57:1563–1565
29. Kape R, Parniske M, Brandt S, Werner D (1992) Isoliquiritigenin, a strong nod gene- and glyceollin resistance-inducing flavonoid from soyabean root exudate. Appl Environ Microbiol 58:1705–1710
30. Denny TP, Vanetten HD (1983) Tolerance of *Nectria haematococca* MP VI to the phytoalexin pisatin in the absence of detoxification. J Gen Microbiol 129:2893–2901
31. Denny TP, Vanetten HD (1983) Characterisation of an inducible, non-degradative tolerance of *Netria haematococca* MP VI to phytoalexins. J Gen Microbiol 129:2903–2913
32. Giannini Jl, Briskin DP, Holt JS, Paxton JD (1988) Inhibition of plasma membrane and tonoplast H+-transporting ATPases by glyceollin. Phytopathology 70:894–896
33. Boydstron R, Paxton JD, Koeppe DE (1983) Glyceollin: a site-specific inhibitor of electron transport in isolated soyabean mitochondrion. Plant Physiol 72:151–155
34. Hungria M, Phillips DA (1993) Effects of a seed colour mutation on rhizobial nod-gene-inducing flavonoids and nodulation in common bean. Mol Plant Microbe Interact 6:418–422
35. Kapulnik Y, Joseph CM, Phillips DA (1987) Flavone limitations to root nodulation and symbiotic nitrogen fixation in alfalfa. Plant Physiol 84:1193–1196
36. Smit G, Puvanesarajah V, Carlson RW, Barbour WM, Stacey G (1992) *Bradyrhizobium japonicum* nodD I can be specifically induced by soyabean tlavonoids that do not induce the nodY ABCSUIJ operon. J Biol Chem 267:310–318
37. Kosslak RM, Bookland R, Barkei J, Paaren HE, Appelbaum ER (1987) Induction of bradyrhizobium japonicum common nod genes by isoflavones isolated from glycine max. Proc Natl Acad Sci U S A 84:7428–7432
38. Kosslak RM, Joshi RS, Bowen BA, Paaren HE, Appelbaum ER (1990) Strain-specific inhibition of nod-gene induction in *Bradyrhizobium japonicum* by flavonoid compounds. Appl Environ Microbiol 56:1333–1341
39. Morris PF, Savard ME, Ward EWB (1991) Identification and accumulation of isoflavonoids and isoflavone glucosides in soyabean leaves and hypocotyls in resistance responses to *Phytophthora megasperma* f.sp. *glycinea*. Physiol Mol Plant Pathol 39:229–244

40. Hahlbrock K, Grisebach H (1975) Biosynthesis offlavonoids. In: Harbome JB, Mabry TJ, Mabry H (eds) The flavonoids Part 2. Academic Press, New York, pp 866–915
41. Masaoka Y, Kojima M, Suguhara S, Yoshihara T, Koshino M, Ichihara A (1993) Dissolution of ferric phosphate by alfalfa (*Medicago satim* L.) root exudates. Plant Soil 155(156):75–78
42. Essalmani H, Lahlou H (2003) Bioprotection mechanisms of the lentil plant by *Rhizobium leguminosarum* against *Fusarium oxysporum* f. sp. *lentis*. Curr Biol 326(12):1163–1173
43. Mishra RPN, Singh RK, Jaiswal HK, Kumar V, Maurya S (2006) *Rhizobium*-mediated induction of phenolics and plant growth promotion in rice (*Oryza sativa* L.). Curr Microbiol 52:383–389
44. Khaosaad T, Garcia-Garrido JM, Steinkellner S, Vierheilig H (2007) Take-all disease is systematically reduced in roots of mycorhizal barley plants. Soil Biol Biochem 39:727–734
45. Rabie GH (1998) Induction of fungal disease resistance in vicia faba by dual inoculation with *Rhizobium leguminosarum* and vesicular-arbuscular mycorrhizal fungi. Mycopathologia 141:3
46. Tu JC (1979) Evidence of deferential tolerance among some root rot rhizobial parasitism in vitro. Physiol Plant Pathol 14:171–177
47. Antoun H, Beauchamp CJ, Goussard N, Chabot R, Lalande CR (1998) Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: effects on radishes (*Raphanus sativus* L.). Plant Soil 204:57–67
48. Malajczuk N, Pearce M, Lichfield RT (1984) Interaction between *Phytopythora cinnamoni* and *Rhizobium* isolates. Trans British Mycol Soc 82:491–500
49. Buonassissi AJ, Copeman RJ, Pepin HS, Eaton GW (1986) Effect of *Rhizobium* spp. on *Fusarium solani* f.sp. *phaseoli*. Can J Plant Pathol 8:140–146
50. Savoure A, Maygar Z, Perre M, Brown S, Schultze M, Dudits D, Kondorosi A, Kondorosi E (1994) Activation of the cell cycle machinery and the isoflavonoid biosynthesis by active *Rhizobium melitoti* Nod signal molecules in *Midicago microcallus* suspensions. EMBO J 13:1093–1102
51. Cordier C, Pozo MJ, Barea JM, Gianianazzi-Pearson V (1998) Cell defence response associated with localised and systemic resistance to *Phytophthora parasitica* induced in tomato by arbuscula mycorrhizal fungus. Mol Plant Microbe Interact 11:1017–1028
52. Brimner TA, Boland GJ (2003) A review of the non-target effects of fungi used to biologically control plant diseases. Agric Ecosyst Environ 100:3–16
53. Phillips DA, Kapulnik Y (1995) Plant isoflavonoids, pathogens and symbionts. Trends Microbiol 3:58–64
54. Eheshamul-Haque S, Ghaffar A (1993) Use of rhizobia in the control of root rot disease of sunflower, okra, soyabean and mungbean. J Phytopathol 138:157–163
55. Arfaoui A, Sifi B, Boudabaous A, Hadrami IE, Cherif M (2006) Identification of *Rhizobium* isolates possessing antagonistic activity against *Fusarium oxysporium* f.sp. *ciceris*, the causal agent of *Fusarium* wilt of chickpea. J Plant Pathol 88:67–75
56. Ruan Y, Kotraiah V, Straney DC (1995) Flavonoids stimulate spore germination in *Fusarium solani* pathogenic on legumes in a manner sensitive to inhibitors of cAMP-dependent protein kinase. Mol Plant Microbe Interact 8:929–938
57. Steinkellner S, Lendzemo V, Langer I, Schweiger P, Khaosaad T, Toussaint JP, Vierheilig H (2007) Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic plant-fungus interactions. Molecules 12:1290–1306
58. Tu JC (1978) Protection from severe phytophthora root rot by *Rhizobium*. Physiol Plant Pathol 12:233–240
59. Husain S, Ghaffar A, Aslam M (1990) Biological control of *Macrophomina phaseolina* charcoal rot sunflower and mungbean. J Phytopathol 130:157–160
60. Chao WL (1990) Antagonistic activity of *Rhizobium* spp. against beneficial and plant pathogenic fungi. Lett Appl Microbiol 10:213–215
61. Huang HC, Kodama F, Akashi K, Konno K (2002) Impact of crop rotation on soilborne diseases of kidney bean: A case study in northern Japan. Plant pathol Bull 11:87–96
62. Matiru VN, Dakora FD (2004) Potential use of rhizobial bacteria as plant growth for increased yield in landraces of African cereal crops. Afr J Biotechnol 3(1):1–7

63. Curir P, Marchesini A, Danielit B, Mariani F (1996) 3-Hydroxyacetophenone in Carnations is a Phytoanticipin active against *Fusarium oxysporum* f. sp. D*ianthi*. Phytocheraistr 41(2):447–450
64. Dakora FD, Vincent JM (1984) Fast-growing bacteria from nodules of cowpea (*Vigna unguiculata* (L.) Walp.). J Appl Bacteriol 56:327–330
65. van Rensburg J, Strijdom H, Kriel MM (1976) Necessity for seed inoculation of soybeans in South Africa. Phytophylactica 8:91–96
66. Ververidis F, Trantas E, Douglas C, Vollmer G, Kretzschmar G, Panopoulos N (2007) Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part II: Reconstruction of multienzyme pathways in plants and microbes. Biotechnol J 2:1235–1249
67. Dixon RA, Paiva NL (1995) Stress-Induced Phenylpropanoid Metabolism. Plant Cell 7(7):1085–1097
68. Vogt T, Pollak P, Tarlyn N, Taylor LP (1994) Pollination-or Wound-lnduced Kaempferol Accumulation in Petunia Stigmas Enhances Seed Production. The Plant Cell 6:11–23
69. Sprent JI (2008) 60 Ma of legume nodulation. What's new? What's changing? J Exp Bot 59(5):1081–1084
70. Dakora FD, Keya SO (1997) Contribution of legume nitrogen fixation to sustainable agriculture in sub-Saharan Africa. Soil and Biochem 29:809–817
71. Dakora FD, Phillips DA (1996) Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins. Physiol Mol Plant Pathol 49:1–20
72. Dakora FD (2000) Commonality of root nodulation signals and nitrogen assimilation in tropical grain legumes belonging to the tribe Phaseoleae. Aust J Plant Physiol 27:885–892
73. Hungria M, Joseph CM, Phillips DA (1991) Rhizobium nod-gene inducers exuded naturally from roots of common bean (Phaseolus vulgaris L.). Plant Physiol 97:759–764
74. Dakora FD and Muofhe ML (1996) Molecular signals involved in nodulation of the African Bambara groundnut. In: Proceedings of the International Bambara Groundnut Symposium, University of Nottingham, UK pp 171–179
75. Messens E, Geelen D, van Montagu M, Holsters M (1991) 7,4-Dihydroxyflavanone is the major Azorhizobium nod-gene-inducing factor present in Sesbania rostrata seedling exudate. Mol Plant-Microbe Interact 4:262–267
76. Haby VA, Stout SA, Hons FM, Leonard AT (2006) Nitrogen fixation and transfer in a mixed stand of alfalfa and Bermuda grass. Agron J 98:890
77. Unkovich MJ, Pate JS (2000) An appraisal of recent field measurements of symbiotic N2 fixation by annual legumes. Field Crops Res 65:211–228
78. Maskey SL, Bhattarai S, Peoples MB, Herridge DF (2001) On-farm measurements of nitrogen fixation by winter and summer legumes in the Hill and Terai regions of Nepal. Field Crops Res 70:209–221
79. Azpilicueta CE, Zawoznik MS, Tomaro ML (2004) Phytoalexins synthesis is enhanced in groundnut plants inoculated with Bradyrhizobium sp.(Arachis). Crop Prot 23:1069–1074
80. De Rijke E. Aardenburg L, Van Dijk J, Ariese F, Ernst WHO, Gooijer C, Brinkman UA (2005). Changed isoflavone levels in red clover (Trifolium pratense L) leaves with disturbed root nodulation in response to water logging. J Chem Ecol 31(6):1285–1298
81. Russell GB, Sutherland ORW, Huchins RFN, Christmas RE (1978) Vestitol: A phytoalexin with insect feeding-deterrent activity. J Chem Ecol 4:571–579
82. Rich JR, Keen NT, Thomason IJ (1977) Association of coumestans with the hypersensitivity of Lima bean roots to Pratylenchus scribneri. Physiol Plant Pathol 10:105–116
83. Fukami H, Nakajima M (1971) Rotenone and the rotenoids. In: Jacobson M, Crosby DG (eds) Naturally Occurring Insecticides. Dekker, New York, pp 71–97