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Review Article

Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins

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Leguminous plants defend themselves against microbial pathogens and herbivores through tissue accumulation of polycyclic compounds before, during, and after attack. Phytoalexins and phytoanticipins, of which isoflavonoids form a major part, are traditionally the compounds used in defence. But some isoflavonoid compounds which classically inhibit pathogens, also serve as chemoattractants, promoters of microbial growth and inducers of nodulation genes in *Rhizobium* and *Bradyrhizobium* bacteria. These newly found biological functions of isoflavonoids clearly complicate the traditional definitions of phytoalexins and phytoanticipins. The release of classical phytoalexins following legume infection with mutualistic microbial symbionts, and the role of these molecules as integrative signals within plants, further confounds the phytoalexin/phytoanticipin concept. This review summarizes some biological roles for isoflavonoid molecules produced in legumes in response to mutualistic symbioses with consideration of the anti-microbial definition of phytoalexins.

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I. INTRODUCTION

Isoflavonoids are a varied group of polycyclic compounds that occur widely in legumes (Fig. 1). Plant pathologists first defined a molecular function for these compounds when they were identified as part of a broader class of anti-microbial molecules known as phytoalexins [91]. Because initial discoveries commonly affect subsequent perceptions, plant pathologists have continued to focus on isoflavonoids as leading examples of phytoalexins, even as they struggle with an ever-changing concept of exactly what comprises a phytoalexin [76, 100]. Most recently, a new term, phytoanticipin, has been proposed [129] to separate traditional phytoalexins, which are formed in response to attacks by pathogens, from chemically identical molecules that are stored in plant cells in anticipation of pathogenic attack. Isoflavonoids comprise a major portion of such phytoanticipins in legumes. One complicating aspect of the phytoanticipin concept is that isoflavonoids serve many functions in addition to inhibiting pathogens. This review supports efforts of others to clarify the term phytoalexin by documenting additional functions in plants for isoflavonoids, the most widely studied class of phytoalexin.

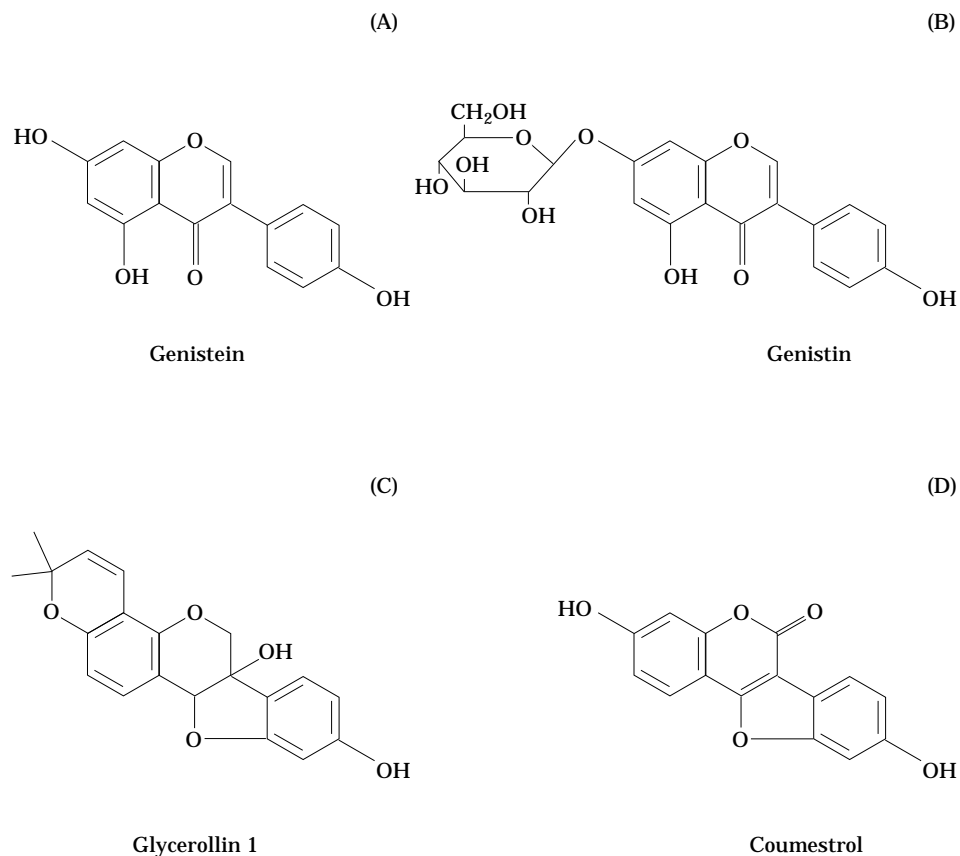


FIG. 1. Representative isoflavonoids from soybean. (A), Genistein and (B), genistin are *nod*-gene inducers [119] which serve as precursors for the phytoalexin (C), glyceollin [48]. Genistin, a constitutive storage product in soybean roots which is hydrolysed and converted to glyceollin in the presence of *Phytophthora megasperma* [48], would be considered a phytoanticipin [129]. (D), Coumestrol is a coumestan isoflavonoid.

II. PHYTOALEXIN DEFINITIONS

Müller and Börger [90] proposed the term phytoalexin to describe a chemical molecule produced by potato tubers in response to attempted infection by *Phytophthora infestans* infection. Based on subsequent studies with common bean, Müller [88] defined phytoalexins as “antibiotics which ... inhibit the growth of micro-organisms pathogenic to plants”. Harborne and Ingham [51] later modified that definition and referred to phytoalexins as, “anti-fungal compound(s) ... produced *de novo* by a plant in response to infection or attempted invasion by fungi or other micro-organisms or treatment with abiotic agents such as toxic chemicals and ultraviolet light”. Subsequently, phytoalexins were defined as low molecular weight, anti-microbial compounds that are both synthesized by and accumulated in plants after exposure to micro-organisms [100]. Most recently, a distinction has been made between traditional phytoalexin compounds

formed *de novo* by a plant under attack from pathogens and phytoanticipins which are characterized as preformed antibiotic compounds stored in plant cells [129]. While such a distinction may be biologically important, its utility is open to question because in many cases phytoalexins and phytoanticipins are identical isoflavonoid molecules.

As that chronology implies, plant pathologists have sought actively to maintain phytoalexins as a viable concept central to plant-microbe interactions. In the 55 years since phytoalexins were first described, however, new data have repeatedly complicated definitions of this concept. For example, early emphasis on anti-fungal, as opposed to general anti-microbial, activity obscured facts about the isoflavonoid coumestrol (Fig. 1D). This compound is produced by cowpea in response to *Phytophthora vignae*, by common bean and soybean in interactions with *Pseudomonas* spp., and by lima bean in response to the nematode *Pratylenchus scribneri* (Table 1). However, despite the fact that this isoflavonoid exhibits both nematicidal and anti-bacterial activity [79, 106], it was not considered a phytoalexin [51] due to its poor fungitoxic properties [127].

What then is a phytoalexin? Day [25] listed bacteria, fungi, viruses, nematodes, and insects as some of the common parasites of plants against which phytoalexins are produced (Table 1). Plants which are partially or wholly dependent on other plants for their nutrition and growth, such as *Striga* on sorghum, or *Alectra vogelii* on cowpea, also qualify as important parasites of agricultural crops against which chemical substances are synthesized by the host plant. Thus, the term phytoalexin has been broadened to include substances formed by host tissue in response to injury, physiological stimuli, infectious agents or their products, that accumulate to levels which inhibit the growth of micro-organisms [76]. The exclusion of nematodes, insects, and other herbivores in the latter definition led Day [25] to further expand the definition of phytoalexins to include substances that are formed in response to infestations that are toxic to, or which repel, insect and nematode parasites, and to some extent herbivores which parasitize plants. As secondary metabolites, phytoalexins have also been defined as compounds which are not obviously essential to the growth and metabolism of the producing organism [80].

Simple isoflavones such as daidzein, genistein (Fig. 1A), biochanin A, formononetin and glycitein, which occur commonly in legumes, are regarded generally as precursors of phytoalexins. These compounds, however, can inhibit the growth of micro-organisms, and are therefore phytoalexins by traditional definition. Daidzein inhibits the growth of *Fusarium culmorum*, while glycitein and formononetin can reduce mycelial development in *Aspergillus ochraceus* [75]. Another study [127] found that formononetin was as good or better an inhibitor of mycelial growth in *Aphanomyces euteiches* and *Fusarium solani* f.sp. *cucurbitae* as several classical phytoalexins. In addition the antifungal compounds from red clover [9], biochanin A and genistein exhibit antifungal activity against *Rhizoctonia solani* and *Sclerotium rolfsii* [133] as well as *Cercospora beticola* and *Monilinia fructicola* [61]. Given the widespread antifungal activity of simple isoflavones, these compounds certainly qualify as phytoalexins.

A complicating issue for the phytoalexin concept has arisen with recent discoveries that many isoflavonoids stimulate infection of plant roots by beneficial microbes and that mutualistic bacteria elicit the exudation of some classical phytoalexins [21]. For example, daidzein, genistein, and coumestrol serve as signal molecules during the establishment of the symbioses between soybean and *Bradyrhizobium*, and between

TABLE 1
Representative isoflavonoid phytoalexins in legume-microbe interactions

Legume	Infected tissue	Inducing microbe	Phytoalexin	Reference
Fungal interactions				
French bean <i>Phaseolus vulgaris</i>	pod	<i>Phytophthora infestans</i>	phaseollin	89
		<i>Rhizoctonia solani</i>	kievitone	120
Pea <i>Pisum sativum</i>	hypocotyl epicotyl	<i>Penicillium expansum</i>	coumestrol	105
		<i>Fusarium solani</i>	pisatin	102
Cowpea <i>Vigna unguiculata</i>	leaf	<i>Collectotrichum lindemuthianum</i>	medicarpin	77
		<i>Phytophthora vignae</i>	coumestrol	99
	leaf		daidzein	99
Faba bean <i>Vicia faba</i>	pod	<i>Botrytis</i> spp.	medicarpin	54
Alfalfa <i>Medicago sativa</i>	leaf	<i>Aschochyta imperfecta</i>	coumestrol	93
			formononetin	93
			daidzein	93
Bacterial interactions				
French bean <i>Phaseolus vulgaris</i>	leaf	<i>Pseudomonas</i> spp.	coumestrol	79
		<i>Pseudomonas phaseolicola</i>	coumestrol	43
			kievitone	
Soybean <i>Glycine max</i>	leaf	<i>Pseudomonas glycinea</i>	coumestrol	67
			daidzein	67
			glyceollin	67
Viral interactions				
Cowpea <i>Vigna unguiculata</i>	leaf	Tobacco necrosis virus	kievitone	1
Pea <i>Pisum sativum</i>	leaf	Tobacco necrosis virus	pisatin	1
French bean <i>Phaseolus vulgaris</i>	leaf	Tobacco necrosis virus	phaseollin	2
			kievitone	10
Nematode interactions				
Lima bean <i>Phaseolus lunatus</i>	leaf	<i>Pratylenchus scribneri</i>	coumestrol	106
Soybean <i>Glycine max</i>	leaf	<i>Meloidogyne incognita</i>	glyceollin	66
Soybean <i>Glycine max</i>	root	<i>Heterodera glycines</i>	glyceollin	58

Phaseolus and *Rhizobium*. These three isoflavonoids specifically induce the transcription of nodulation (*nod*, *nol*) genes in *Bradyrhizobium japonicum* [73] and *Rhizobium leguminosarum* bv. *phaseoli* [22, 59]. So, clearly, some isoflavone phytoalexins act as positive signals to mutualistic rhizobia, thus confusing traditional phytoalexin definitions. These findings do not justify abandoning the phytoalexin concept, but do, indicate that a major category of phytoalexins, the isoflavonoids, are involved in important biological processes that transcend traditional anti-microbial definitions of these compounds.

III. OCCURRENCE OF ISOFLAVONOID PHYTOALEXINS IN LEGUMES

Many isoflavonoid phytoalexins have been chemically identified in legumes and catalogued by various workers [32]. Non-flavonoid phytoalexins from diverse chemical groups including stilbenes, benzofurans and furanoacetylenes, have also been characterized [51], but they will not be mentioned further in this discussion. Isoflavonoids occur as both simple aglycones and as glycosidic conjugates (Fig. 1B) in many legume genera, but members of the subfamily Papilionoideae of

the Leguminosae contain the largest number of these compounds. Isoflavonoid phytoalexins fall into numerous chemical subclasses, including isoflavones, isoflavanones, pterocarpans, isoflavans, and coumestans, and it is common to find more than one type in a single plant species. In some cases, the same isoflavonoids are produced by different species as in *Vigna unguiculata* and *Phaseolus vulgaris* which both make daidzein, kievitone, and coumestrol (Table 1), as well as several related compounds.

Substantial quantities of isoflavonoids occur in apparently healthy seeds, roots, and shoots of legumes [60]. From a phytopathological point of view, any part or organ of a plant can be the site of isoflavonoid accumulation if it is attacked by a pathogen. In soybean, daidzein, genistein, and their glycosidic conjugates are present in the embryo, cotyledons, hypocotyl and seedling roots [46]. While a similar mixture of isoflavonoids was found in most soybean organs studied in that work [46], alfalfa isoflavonoids have been shown to differ between the root and shoot [69, 70]. Organized plant tissues are not the only source of isoflavonoids in some systems. Root exudates contain isoflavonoids [10, 21, 22, 114], and those compounds may come from sloughed-off root cap cells, referred to as root border cells, which are released in large numbers [57]. The biosynthetic potential of plant tissues may not necessarily be reflected directly by the isoflavonoids identified in various studies. Factors of availability and chemical concentration strongly influence natural product chemists, and it is often possible to find lesser amounts of the same molecule in other parts of the same plant. Similarly, other isoflavonoids may have been ignored in the same tissue because they were present in low concentrations or could not be identified by the investigator.

A. ISOFLAVONOIDS IN PATHOGENESIS

Historically, the presence of isoflavonoids has been associated most closely with pathogenic events in legumes. Traditional discussions of phytoalexins have emphasized the *de novo* synthesis of these compounds in response to pathogens (Table 1), and numerous reviews have covered this complex topic [5, 35]. Hydrolysis of pre-existing flavonoid glycosides may also contribute isoflavone molecules that can be converted to more complex pterocarpans in the presence of a pathogen [48]. In either case, localized hypersensitive responses of plants to many pathogenic infections result in the accumulation of isoflavonoids and these responses have some parallels in symbiotic interactions between *Rhizobium* and legumes [78]. In contrast, the current understanding of systemic responses of non-legumes to pathogens does not involve flavonoids [16, 28], but future studies in legumes, where these compounds are more common, may find that isoflavonoids influence both localized and systemic responses.

Stimulation of isoflavonoid biosynthesis in plants is a common feature of many infecting micro-organisms irrespective of whether they are bacteria, fungi, viruses or nematodes (Table 1). Different legume-parasite interactions can cause the accumulation of similar phytoalexin molecules e.g., soybean infection by nematodes, fungi and bacteria all stimulate the release and accumulation of the classical phytoalexin glyceollin (Fig. 1C). Consequently, the commonly observed correlation between inhibition of microbial growth and phytoalexin accumulation [53], the fact that metabolic inhibitors which block phytoalexin accumulation also reduce resistance

[84], and the evidence that transfer of phytoalexins to compatible associations promote incompatibility [68] support the role of phytoalexins in legume immunity to various pathogens and parasites. Also, the demonstration that pathogen ability to degrade host plant phytoalexins is important for pathogenicity [112] is a further indication of the role of phytoalexins in plant disease resistance.

B. ISOFLAVONOIDS IN MUTUALISTIC ASSOCIATIONS

Recent studies have established that isoflavonoids are directly involved in mutualistic symbioses between legumes and soil micro-organisms. *Rhizobium* is generally viewed as a mutualistic symbiont, but early events involved in nodule formation, such as root hair deformation, root hair curling, infection thread formation and cortical cell division, suggest that the organism is a sophisticated parasite [36]. Both bacterial [81] and fungal mutualistic symbionts [55, 132] trigger defense-like reactions in host plants during the establishment of the symbioses. Some of these responses are evident as exudation of isoflavonoids [21, 22, 114], but in the alfalfa-*Rhizobium* symbiosis, morphological evidence shows a hypersensitive-like response in which infection is aborted and phenolic compounds accumulate [130]. Functional symbiotic tissues containing mutualistic fungi [135] or rhizobia [96], generally do not contain high levels of isoflavonoid phytoalexins, but some increases have been detected [85], especially in those cases where a final stage of the symbiosis (e.g. N₂ fixation) is genetically blocked in the microsymbiont [98]. Elicitation of isoflavonoids and signal functions of these molecules in mutualistic associations are discussed more fully in the following sections.

IV. PRODUCTION OF ISOFLAVONOID PHYTOALEXINS

Many detailed studies have examined how pathogens elicit the synthesis of isoflavonoid phytoalexins [35]. Recently it has become apparent that some parallel events occur during mutualistic plant-microbe interactions. VA mycorrhizal fungi [55, 131], *B. japonicum* [40, 115], *R. meliloti* [21, 111], and *R. leguminosarum* bv. *phaseoli* [22], for example, all enhance isoflavonoid production or exudation in their host legume. Further studies may clarify the extent to which these exuded isoflavonoids come from excreted phytoanticipins as opposed to newly synthesized phytoalexins. Such investigations may show that low concentrations of phytoanticipins are released continuously as signals that attract and produce useful microbial communities while higher levels of the same compounds would help combat pathogens.

A. BIOSYNTHESIS, STORAGE AND RELEASE

Isoflavonoids occur in legumes grown under sterile conditions without exposure to any apparent biological or environmental stress factors [124]. In such plants these compounds generally occur as free aglycones, or as glucosides, and malonylated glucosides. Biosynthetic pathways responsible for producing the aglycones are well characterized with the important exception of coumestans [32], and enzymatic reactions associated with glycosylation and malonylation have also been described [5, 6]. Those reports should be consulted for information on the structural and enzymological details involved in synthesis of these compounds. The key issues for

understanding when one of these compounds is a phytoanticipin as opposed to a phytoalexin involve defining whether a micro-organism triggers synthesis and exudation, or hydrolysis of a stored conjugate and exudation.

When plants are exposed to pathogenic or mutualistic symbionts, transcription of genes in the phenylpropanoid (e.g. phenylalanine ammonia-lyase, PAL) and flavonoid (e.g. chalcone synthase, CHS) pathways, which are required for isoflavonoid synthesis, is greatly enhanced. Because these key enzymes are coded for by members of multigene families, however, there is much to be learned about regulatory events that control whether two structurally identical molecules function as phytoalexins and phytoanticipins. Thus PAL and CHS genes are normally being transcribed in alfalfa at low levels (perhaps to produce phytoanticipins?), but different copies of the genes are induced by the pathogenic symbiont *Phoma medicaginis* [62] and the mutualistic symbiont *R. meliloti* [111]. Likewise, in soybean roots, *B. japonicum* cells induce particular subsets of the PAL and CHS gene families [40].

The cellular location of isoflavonoids may offer some hints as to whether particular molecules function as phytoalexins or phytoanticipins. Relatively little is known about cellular compartmentation of isoflavonoids, but a cogent summary of published information on other flavonoids, especially anthocyanins, was offered by Stafford [123]. A logical extension of her discussion suggests that enzymes bound to the endoplasmic reticulum release newly synthesized isoflavonoids and isoflavonoid conjugates into vesicles. The vesicles then fuse either with the vacuole if they contain phytoanticipins or to the plasma membrane if they contain phytoalexins. In this model, the membrane carrier, not the isoflavonoid, determines the fate of structurally identical molecules. At this level of discussion, the ultimate function of the isoflavonoid molecule has little relevance to understanding the process. The key factor, which indeed is already embodied in the concepts of phytoalexins and phytoanticipins, is that identical molecules serve varying purposes by being released at different times or in differing concentrations.

Following this line of thinking, one can better understand how soybean seedlings might use daidzein and genistein as both phytoalexins and phytoanticipins. These two isoflavones are synthesized constitutively as phytoanticipins and stored in the conjugated form [48]. However, with elicitation by bradyrhizobial Nod factors [115] or by the pathogen *Phytophthora megasperma* f.sp. *glycinea* [87], larger amounts of molecules are synthesized by enzymes controlled by microbially responsive promoters. Because synthesis has been triggered by microbial factors, the active enzymes may be associated with membranous vesicles that fuse to the plasma membrane and release genistein or glyceollin outside the plant cell [45, 48].

B. TRIGGERING ISOFLAVONOID PRODUCTION WITH ELICITORS

While differences among phytoalexins, phytoanticipins, and signal isoflavonoids may be reflected at the level of gene transcription and cellular packaging, earlier steps in the plant-microbial interaction may also affect such processes. The earliest steps undoubtedly involve microbial products that induce (i.e. elicit) signalling pathways and result in isoflavonoid production (Table 3). Abiotic stresses (Table 4) that elicit the same or different transduction pathways also are known. In fungi, these

biotic elicitors are specific compounds like chitosan (β -1,4-glucosamine) and β -1,3-glucans (Table 3). It is now clear that most biotic elicitors consist mainly of carbohydrates from microbial cell walls, although proteins and lipids are also involved. A complex carbohydrate compound, β -1,3-1,6-oligoglucoside isolated from the hyphal walls of *Phytophthora megasperma* f. sp. *glycinea* was the first molecule to be found with elicitor activity [116]. Following that observation, many elicitor molecules have been purified from pathogens. Host plant cell walls also produce phytoalexin-inducing elicitors of carbohydrate origin. Soybean polysaccharides such as oligo- α -1,4-galacturonide induce glyceollin phytoalexins in the soybean plant itself after injury [92]. Details on the types and functioning of oligosaccharide elicitors from pathogens and host plants can be found in an authoritative review by Hahn *et al.* [50].

TABLE 2
Examples of legume isoflavonoids affecting symbiotic interactions

Isoflavonoids	Legume species	Symbiotic role	Reference
Aglycones			
Daidzein	Soybean <i>Glycine max</i>	<i>nod</i> gene inducer	73
Genistein	Soybean <i>Glycine max</i>	<i>nod</i> gene inducer	73
Coumestrol	Soybean <i>Glycine max</i>	<i>nod</i> gene inducer	73
Daidzein	French bean <i>Phaseolus vulgaris</i>	<i>nod</i> gene inducer	22
Genistein	French bean <i>Phaseolus vulgaris</i>	<i>nod</i> gene inducer	59
Coumestrol	French bean <i>Phaseolus vulgaris</i>	<i>nod</i> gene inducer	22
Daidzein	White clover <i>Trifolium repens</i>	<i>nod</i> gene inhibitor	37
Biochanin A	White clover <i>Trifolium repens</i>	<i>nod</i> gene inhibitor	37
Formononetin	White clover <i>Trifolium repens</i>	<i>nod</i> gene inhibitor	37
Formononetin	Soybean <i>Glycine max</i>	<i>nod</i> gene inhibitor	74
Biochanin A	Soybean <i>Glycine max</i>	<i>nod</i> gene inhibitor	74
Daidzein	Soybean <i>Glycine max</i>	resistance inducer	97
Genistein	Soybean <i>Glycine max</i>	resistance inducer	97
Formononetin	White clover <i>Trifolium repens</i>	hyphal growth	117
Biochanin A	White clover <i>Trifolium repens</i>	hyphal growth	117
Formononetin	Alfalfa <i>Medicago sativa</i>	spore inhibitor	126
Conjugates			
Genistin	Soybean <i>Glycine max</i>	<i>nod</i> gene inducer	119
6"-O-malonyldaidzin	Soybean <i>Glycine max</i>	<i>nod</i> gene inducer	119
6"-O-malonylgenistin	Soybean <i>Glycine max</i>	<i>nod</i> gene inducer	119
Formononetin-7-O-(6"-O-malonylglucoside)	Alfalfa <i>Medicago sativa</i>	<i>nod</i> gene inducer	21

Mutualistic bacteria produce elicitors like fungal glucans as well as other lipooligosaccharides, which, because of their involvement in root nodule initiation, are termed Nod factors [29] (Table 3). Cyclic β -1,6-1,3-glucans synthesized by both free-living cells and bacteroids of *B. japonicum* [44, 82] are active elicitors of

TABLE 3
Representative microbial elicitors of phytoalexins in legumes

Elicitor	Source	Phytoalexin	Host	Reference
Pathogen				
β-1,3-1,6-oligo-glucoside	<i>P. megasperma</i>	Glyceollin	Soybean <i>Glycine max</i>	116
β1,3-glucan	<i>P. megasperma</i>	Glyceollin	Faba bean <i>Vicia faba</i>	38
β-1,4-glucosamine	<i>P. megasperma</i>	Pisatin	Pea <i>Pisum sativum</i>	
Mutualistic symbiont				
β-1,6-1,3-glucan	<i>B. japonicum</i>	Glyceollin	Soybean <i>Glycine max</i>	83
β-1,6-1,3-glucan	<i>B. japonicum</i>	Daidzein	Soybean <i>Glycine max</i>	83
Purified Nod factor	<i>B. japonicum</i>	Coumestrol	Soybean <i>Glycine max</i>	115
		Daidzein		
		Genistein		
Purified Nod factor	<i>R. NGR234</i>	Coumestrol	Soybean <i>Glycine max</i>	115
		Daidzein		
		Genistein		

glyceollin in soybean [83]. Whether these molecules bind to the same receptor as the glucans from pathogens is not known. In soybean roots, the formation and exudation of daidzein, genistein and coumestrol can be elicited by purified Nod factors from *B. japonicum* [115]. Unlike β-glucans, however, Nod factors alone do not elicit the exudation of glyceollin. Those observations suggest that the Nod factor does not stimulate *de novo* synthesis, but rather promotes constitutive pathways of isoflavonoid biosynthesis. Higher levels of elicitation may be prevented by legume root chitinase inactivation of Nod factors [122]. Mutualistic VA mycorrhizal fungi also show what might be classified as a partial hypersensitive response in alfalfa roots where steady-state levels of PAL, CHS and chalcone isomerase (CHI) transcripts increase rapidly and then decline precipitously [132]. Although details of exactly how mutualistic symbionts evade plant phytoalexin responses remain to be described, it is apparent that many identical or parallel molecular pathways are activated by both pathogens and mutualists.

The mechanisms by which abiotic factors (Table 4) affect isoflavonoid phytoalexin formation in plants are unclear. Because these factors have diverse structural features, they may act simply by injuring plant cells, which then stimulates the phytoalexin biosynthetic pathway, or they may cause the host plant to release a constitutive elicitor which triggers phytoalexin formation [121]. Although abiotic elicitors, such as low concentrations of specific nutrients in soil, can affect the formation of isoflavonoids in legumes, the physiological role of this phenomenon has not been assessed. Without considering soil factors, plant ecologists often contend that the accumulation of isoflavonoids and other phenolics in plant tissues serve an anti-herbivore function. Yet an intrinsically low level of many nutrient elements (Table 4) might stimulate isoflavonoid production irrespective of whether herbivores are present, or not.

Whether an elicitor is a fragment of a cell or a defined chemical molecule such as a β-glucan, host plants must recognize these phytoalexin-inducing factors. Such molecules from both pathogens and mutualists are presumably recognized by receptors located

TABLE 4
Representative abiotic factors inducing phytoalexin production in legumes

Abiotic stress	Phytoalexin	Host tissue	Reference
Low N	Coumestrol	Soybean (<i>G. max</i>) root	18
	Daidzein		
	Genistein		
Low P	Biochanin A	Subterranean clover (<i>Trifolium subterraneum</i>) leaf	108
	Formononetin		
	Genistein		
Low S	Biochanin A	Subterranean clover (<i>T. subterraneum</i>) leaf	109
	Formononetin		
	Genistein		
Low Ca	Daidzein	Soybean (<i>G. max</i>) root	134
	Glyceollin		
High Mg	Daidzein	Soybean (<i>G. max</i>) root	134
	Glyceollin		
FeSO ₄	Glyceollin	Soybean (<i>G. max</i>) hypocotyl/radicle	26
CuCl ₂	Glyceollin	Soybean (<i>G. max</i>) hypocotyl/radicle	26
HgCl ₂	Daidzein	Soybean (<i>G. max</i>) cotyledons	47
	Genistein		
	Glyceollin		
AgNO ₃	Daidzein	Soybean (<i>G. max</i>) cotyledons	47
	Genistein		
	Glyceollin		
Ethylene	Pisatin	Pea (<i>P. sativum</i>) pods	15
Iodoacetate	Glyceollin	Soybean (<i>G. max</i>) cotyledons	47
UV	Coumestrol	Soybean (<i>G. max</i>) cotyledons	47
	Glyceollin		
Detergent TX-100	Coumestrol	Soybean (<i>G. max</i>) cotyledons	47
	Genistein		
	Glyceollin		
High light	Biochanin A	Subterranean clover (<i>T. subterraneum</i>) leaf	107
	Formononetin		
	Genistein		

in the plasma membrane. Legumes have specific receptors for particular elicitors [50] and accept rhizobial bacteria as mutualists only if they recognize the Nod factors secreted by that strain [29]. The soybean-*Bradyrhizobium* interaction offers one example of the complexity of elicitor perception by host plants. Soybean-nodulating bacteria form two classes of known elicitors that affect the legume host: (1) cyclic β -1,6-1,3-glucans from bradyrhizobia, which stimulate glyceollin biosynthesis in soybean [33]; and (2) Nod factors which trigger the formation of daidzein, genistein and coumestrol, but not glyceollin, in the soybean plant [115]. As host, the soybean must recognize two differing elicitors from its bacterial symbiont to produce the varying phytoalexin responses. Because receptors in the soybean plasma membrane are highly specific for the oligoglucoside elicitors they bind [50], these elicitors may interact with separate receptors. As yet, however, no specific receptors have been identified.

Although our understanding of elicitor-receptor interactions is limited in plant-microbe associations, legume perception of Nod factor elicitors involves the nature of the chemical moiety attached to the reducing end of the Nod factor molecule. While

acylation of a *B. japonicum* Nod factor can enhance its recognition by a host soybean plant and its efficacy at eliciting phytoalexin formation, hydroxylation causes an altered perception of the Nod factor as elicitor, leading to increased synthesis of only daidzein and coumestrol [115]. Sulphation almost completely eliminates the elicitation potency of the same Nod factor. In alfalfa, however, sulphation of the Nod factor is required for effective nodulation by *R. meliloti* [125].

A recent report suggests that tomato cell cultures can perceive Nod factors from *Rhizobium* and *Bradyrhizobium* bacteria [122]. This observation raises several questions. Do legumes and non-legumes recognize the elicitor by the same mechanism? Do non-legumes perceive cyclic β -1,6-1,3-glucan elicitors of bradyrhizobial origin? Does tomato, like legumes, recognize bacterial Nod factors and produce isoflavonoids? Essentially no information is available on the transduction pathway(s) between hypothetical elicitor receptors and the carefully documented effects of microbes on gene families responsible for synthesis of phenylpropanoid, flavonoids, and isoflavonoids [34]. If these pathways are highly conserved, then much of the specificity of plant-microbe interactions should reside in the initial recognition of the elicitor.

V. SIGNALLING FUNCTIONS OF ISOFLAVONOIDS

It is now well-established that mutualistic bacteria and fungi, pathogenic microbes, and insect pests can elicit isoflavonoid phytoalexins in legumes [11, 21, 22, 52, 114, 115]. Functions of these compounds have not all been defined at the molecular level, but in many cases newly elicited isoflavonoid signals regulate microbial genes that promote the development of mutualistic symbioses. The release of traditional isoflavonoid phytoalexins under conditions less threatening to the plant than general pathogenic attack goes to the heart of the problem created by labelling molecules according to their biological function rather than by their chemical structure. A brief summary of biological activities reported for isoflavonoids is offered below to indicate how isoflavonoid compounds that fall into the functional category proposed for phytoanticipins, actually play many biological roles not currently included in that definition.

A. TRANSCRIPTIONAL REGULATION OF NODULATION GENES

The best understood signalling functions of isoflavonoids involve transcriptional regulation of *nod* genes in mutualistic rhizobia and bradyrhizobia (Table 2). The first study in this area found that daidzein, genistein and coumestrol from soybean roots induce transcription of the *nod* genes in *B. japonicum* [73]. Subsequent work showed that significant strain differences occurred in the chemical specificity of this regulation [74], and other workers identified isoflavonoid aglycones that are released from host legumes and induce *nod* genes in different species of rhizobial symbionts [22, 59]. In addition, isoflavonoid conjugates, which are present in soybean and fall under the definition of phytoanticipins, induce *nod* genes in the soybean symbiont, though through a separate regulatory system [119]. Parallel observations found that alfalfa root exudates contain formononetin-7-O-(6"-O-malonylglycoside), which activates *nod* gene expression in *R. meliloti* [21].

Some isoflavonoids play molecular roles beyond the enhancement of *nod*-gene transcription. Daidzein, for example, regulates expression of two genetic loci apparently unrelated to *nod* genes [110]. Also, several reports indicate that isoflavonoids can inhibit *nod*-gene induction under laboratory conditions [37, 41, 74]. No evidence yet documents the ecological relevance of such responses. Presumably the effects result from structural similarities between an inducer and inhibitor which compete for binding to an active site. While it is possible to inhibit root nodule formation by *B. japonicum* USDA110 on soybean as much as 96% with 20 μM 7-hydroxy-5-methylflavone [19], natural variation in the responses of *B. japonicum* strains in soils probably will prevent this strategy from being used to favour genetically improved bacteria [19, 74].

It is likely that during the establishment of mutualistic plant-microbe associations, the bacteria and fungi encounter toxic levels of isoflavonoid molecules in the rhizosphere. It has long been known that rhizosphere bacteria catabolize formononetin [4]. Rhizobia degrade daidzein and genistein [104], but no information is available on whether those reactions are constitutive or inducible. In *Nectria haematococca*, the important contribution of pisatin degradation to pathogenesis was proven by genetic techniques, which showed that the presence of pisatin demethylase allowed the fungus to degrade this phytoalexin and infect pea [113]. Studies in non-pathogenic organisms may well demonstrate that the capacity to detoxify isoflavonoids is a defining trait in most rhizosphere micro-organisms [101].

B. CHEMOATTRACTANTS

One important aspect of plant-microbe interactions is a positive chemotaxis, which is often exhibited by a micro-organism in the presence of the host plant. The isoflavonoids daidzein and genistein from soybean root exudates, for example, attract zoospores of the pathogen, *Phytophthora sojae* [86]. Similarly, the isoflavones isoformononetin and biochanin A attract zoospores of the soybean pathogen *Phytophthora sojae* and the alfalfa pathogen *Phytophthora cryptogea* [86]. Chemotaxis also contributes to development of mutualistic rhizobial associations [13]. Flavonoids released from alfalfa produce a positive chemotaxis in *R. meliloti* [12, 33]. Studies with *B. japonicum* showed that, although soybean seed rinses and root exudates attract *B. japonicum* [3], the isoflavonoids daidzein, genistein and coumestrol are not the active compounds [3, 64].

C. GROWTH EFFECTS

Isoflavonoids have definite effects on growth of fungi (Table 2) and possibly bacteria. Micromolar concentrations of daidzein stimulate germination of *Glomus* spores [65], but similar levels of formononetin significantly inhibited that process in two *Glomus* species [126]. In a similar *in vitro* system, biochanin A inhibited the growth of *G. margarita* hyphae when the compound was supplied after spore germination [7]. By contrast, higher concentrations of formononetin or biochanin A stimulated hyphal growth of *Glomus* on white clover [117]. The precise concentrations of these compounds that would be available under natural conditions are not known, so it is impossible to predict which, if any, of these processes occur in nature. Formononetin and glyceitin also stimulate hyphal growth of *Penicillium digitatum* and *Fusarium culmorum* [75], two

fungi that often are associated with food contamination. The record of isoflavonoid effects on promoting bacterial growth is less convincing. The flavonoids luteolin and quercetin have very definite promotive effects on growth of *R. meliloti* in a minimal medium [56], but the isoflavones daidzein and genistein gave confusing results in similar tests with *B. japonicum* [24].

D. HOST PLANT PROTECTANTS

One can appropriately suggest that isoflavonoids included in the original definition of phytoalexins were signals in the sense that they induced microbial responses required for resistance [30, 31]. Because a major portion of that resistance involves enzymatic degradation of the phytoalexin [128], it is generally assumed that phytoalexins induce the catabolic pathway responsible for their degradation. Thus, for example, the legume isoflavonoid phytoalexins, medicarpin and macckiain, are degraded by the common legume pathogens *Nectria haematococca* and *Ascochyta rabiei* via NADPH-dependent reductase conversion of the molecule to a less toxic isoflavan, isoflavanone, or 6a-hydroxypterocarpan [128]. Likewise the bean pathogen, *Fusarium solani* f. sp. *phaseoli*, can detoxify four major bean phytoalexins, kievitone, phaseollin, phaseollidin, and phaseollinisoflavan. Kievitone detoxification occurs through kievitone hydratase catalysed hydration of the isopentenyl side chain to yield the less toxic kievitone hydrate [128].

Less is known about how micro-organisms involved in mutualistic associations respond to isoflavonoid phytoalexins. Experiments showed that *Rhizobium* and *Bradyrhizobium* cells were sensitive to some but not all of 11 isoflavonoids tested [95]. Kievitone and medicarpin were generally the strongest inhibitors of growth [95, 118], but not all rhizobial species were affected. Pisatin, coumestrol, biochanin A, formononetin, genistein, rotenone and vestitol produced no inhibitory effect. No chemical analyses were conducted to measure whether the bacteria used catabolic reactions to protect against the compounds, but other studies show that rhizobia can degrade many flavonoids [103, 104]. No information is available on whether these are constitutive or inducible catabolic processes. An intriguing observation that pre-culturing *B. japonicum* cells with 10 μM genistein or daidzein induced resistance against the phytoalexin glyceollin [97] suggests that transcriptional regulation may be involved in this phenomenon, but no proof is available. The chalcone isoliquiritigenin, a *nod*-gene inducer in *B. japonicum*, also induces resistance to glyceollin [63], but glyceollin resistance has been produced in a mutant lacking the regulatory *nod* genes *nodD₁D₂YABC* [98]. Thus the isoliquiritigenin effect may not be mediated by *nod* genes. It has been suggested that glyceollin is toxic because it inhibits plasma membrane and tonoplast H⁺-transporting ATPases [42] and/or NADH-ubiquinone-oxidoreductase [8], and so no direct involvement of *nod* genes would necessarily be expected.

E. INTERNAL PLANT SIGNALS

Significant quantities of isoflavonoid conjugates are stored in legume roots. In alfalfa, conjugated forms of formononetin, medicarpin, and coumestrol were present in roots at concentrations of 865, 135, and 25 nmoles g fresh weight⁻¹ [124]. While these molecules certainly may represent stored pools available for use against micro-

organisms, they may also integrate tissues and organs within the plant by serving as cell-to-cell signals. Simple analyses of alfalfa xylem sap, for example, show flavonoids are transported from roots to shoots (D. A. Phillips, unpublished data). Isoflavonoid conjugates released from vacuoles of pathogen-infected tissues could easily be transported in the xylem stream to other parts of the plant. Under those conditions, natural selection could favor plants that respond with beneficial changes, such as systemic acquired resistance (SAR). Legumes develop SAR following infection by pathogens [71], and the development of this resistance is preceded by increased isoflavonoid phytoalexin production in pre-inoculated tissues [39]. While there is no indication yet that isoflavonoids are linked to SAR, evidence is accumulating that plants have multiple resistance mechanisms which are linked together by complex interconnected signalling pathways [27].

Plants might also use internal regulatory pathways involving isoflavonoids to respond to beneficial micro-organisms. Root nodulation, for example, is systemically regulated in legumes [14]. When intact roots of a soybean plant are separated into two different compartments, *B. japonicum* inoculation on one side of the split-root system strongly inhibits nodulation on the other [72], and soybean mutants that show hypernodulation are less able to exert a systemic suppression of nodule formation under such experimental conditions [94]. Any actual role of isoflavonoids in such responses remains to be proven, but grafting shoots of hypernodulating soybeans to wild-type roots, significantly increased the levels of daidzein, genistein and coumestrol in the rootstock [17].

VI. CONCLUSIONS

Phytoalexins, including isoflavonoids, are biologically important molecules [20]. Their isolation and identification in the past 50 years have helped scientists develop a central core of knowledge crucial for understanding plant-pathogen interactions. Advances in analytical techniques have complicated the original concept of isoflavonoid phytoalexins by showing that they are normally stored in plants, that they are released at low levels under many conditions when pathogens are not present, and that they regulate how plants interact with beneficial microbes. If new studies show that isoflavonoids function as integrative signals within plants, the scientific importance of isoflavonoids will increase, but the functional significance of phytoalexins will not be diminished. Historically, chemists studied molecular structures, and biologists examined organismic functions. Given those approaches, it was reasonable to have structural definitions in chemistry and functional definitions in biology. Phytoalexins and phytoanticipins have both been defined in functional terms. As chemistry and biology grow closer, it becomes more difficult and less reasonable to defend purely functional or simply structural definitions. To do so, produces artificial partitions between complementary scientific disciplines. Perhaps the clearest avenue to future understanding of isoflavonoids and phytoalexins lies in replacing old definitions with new questions, rather than with new definitions that may constrict thought.

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