MPMI Vol. 8, No. 6, 1995, pp. 929–938. © 1995 The American Phytopathological Society

Flavonoids Stimulate Spore Germination in *Fusarium solani* Pathogenic on Legumes in a Manner Sensitive to Inhibitors of cAMP-Dependent Protein Kinase

Yijun Ruan, Vinayaka Kotraiah,[1] and David C. Straney

Department of Plant Biology, and the Maryland Agriculture Experiment Station, University of Maryland, College Park, MD 20742, U.S.A.; [1]Molecular and Cellular Biology Program, University of Maryland, College Park, MD 20742, U.S.A.

Received 30 May 1995. Accepted 28 August 1995.

Many soilborne fungal plant pathogens remain as resting propagules until the appearance of a potential host stimulates their germination. The plant-derived stimulus for germination has generally been assumed to be nutrients exuded from roots. We show that certain flavonoids, including defense-related isoflavonoid phytoalexins, stimulate spore germination of *Fusarium solani* formae speciales pathogenic on pea or bean. The stimulatory action of specific flavonoids are consistent with the flavonoids previously identified in root exudates of these two hosts and with the levels of flavonoids reported to be exuded by bean roots. Inhibitors of cAMP-dependent protein kinase (PKA) prevented flavonoid-responsive germination, but not nutrient-responsive germination. Thus, these two stimuli, flavonoids and nutrients, appear to utilize separate signal pathways to initiate germination. Germination of macroconidia in root exudates was significantly inhibited by a PKA inhibitor, indicating that flavonoids present in root exudates may be at least as active as nutrients in stimulating germination. These results suggest that flavonoids in legume root exudate may be perceived as a signal in a number of plant-microbe interactions, not only for initiating symbiotic rhizobial interactions but also for initiating pathogenic fungal interactions.

Additional keywords: apigenin, *F. solani* f. sp. *pisi*, genistein, *Nectria haematococca*, PDA1, pisatin.

Most fungi that cause soilborne diseases of plants remain dispersed in soil as resting propagules, such as spores or sclerotia, in the absence of their hosts. Appearance of a potential host stimulates vegetative growth from these propagules, allowing the pathogen to initiate further interaction with the plant. Fungal spores presumably recognize the presence of a plant through soluble or volatile compounds since exudates from germinating seeds and roots stimulate germination of spores from a number of genera, including *Pythium*, *Phytophthora*, and *Fusarium* (Nelson 1991). Although it has been thought for some time that sugars and amino acids exuded from the root serve as a general germination stimulus, recent experiments with *Pythium ultimum* indicate a potentially more limited role for these nutrients in stimulating germination of natural inoculum (Nelson 1991; Nelson and Hsu 1994). The nature of the stimulatory signal and the host specificity of the signal is unknown in most fungi studied (reviewed in Nelson 1991). Elucidation of additional or more specific signals would clarify the initial events and specificity involved in fungal recognition of potential hosts at this initial stage of the interaction.

Fusarium solani is one experimental organism used in early studies on plant-induced germination. This fungus causes foot and root rot on a variety of plants, although many strains display host specificity. Chlamydospores of *F. solani* f. sp. *pisi*, a pea pathogen, germinate after introduction of germinating pea seeds into infested soil. This response occurs within 24 h and within a 7 mm radius of the plant (Cook and Snyder 1965; Short and Lacy 1974). Glucose and amino acids have been proposed to be the potential plant-derived signals which trigger this germination since they stimulate germination and are found in root exudates (Schroth et al. 1963; Short and Lacy 1976). However, a discrepancy between the higher levels of these nutrients required for germination than those present in the rhizosphere had lead to the supposition that the exudates contain other stimulatory substances as well (Short and Lacy 1976). Like most other systems, the nature of other possible stimulatory signals is unknown.

During our study of plant-responsive *PDA* gene regulation in *F. solani* f. sp. *pisi* (strains of the ascomycete teliomorph *Nectria haematococca* Berk. & Br. mating population [MP] VI), we observed that spores of this fungus germinated at a much higher frequency in minimal medium to which a host pterocarpan isoflavonoid was added. This isoflavonoid, pisatin, is a phytoalexin produced by garden pea (*Pisum sativum*) (Pueppke and VanEtten 1976). Pisatin induces expression of the *F. solani* f. sp. *pisi PDA*1 gene which encodes a pisatin demethylase allowing pisatin detoxification (VanEtten et al. 1989). We were intrigued by the stimulatory activity of such a plant-derived flavonoid, since flavonoids have been found in legume root exudates and the rhizosphere in studies regarding their stimulation of nodulation (*nod*) gene expression in most rhizobial bacteria (Fisher and Long 1992; Leon-

Corresponding author: D.C. Straney; e-mail: straney@umail.umd.edu

Present address of Y. Ruan: Monsanto Co., U3D, 800 N. Lindberg Blvd., St Louis, MO 63167 U.S.A.

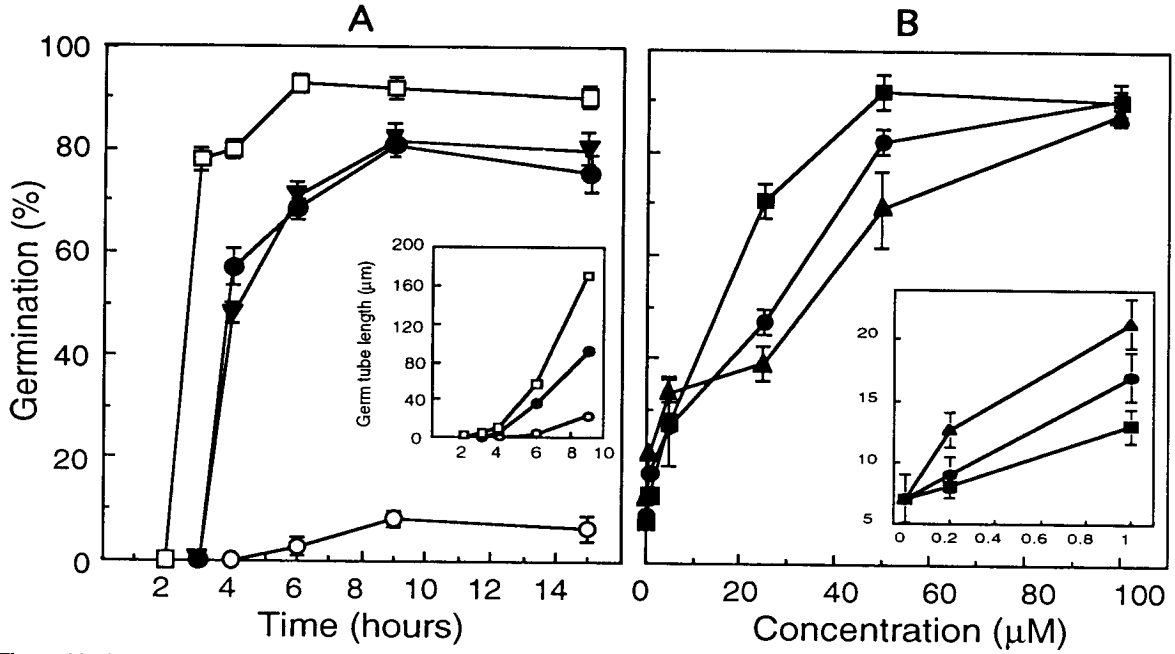

Fig. 1. Flavonoid stimulation of *Fusarium solani* f. sp. *pisi* macroconidia germination. Macroconidia were suspended in growth media at 10^6/ml and growth was quantitated as percent of macroconidia which have germinated.. **A,** The germination over time of incubation is shown for rich medium, □ , and minimal medium, ○ , without additions. The flavanone naringenin, ● , and the pterocarpan isoflavonoid pea phytoalexin pisatin, ▼ , were added to minimal medium at 50 μM concentration. Inset: The average length of germ tubes on macroconidia which had germinated at the specified times is shown. Naringenin and pisatin had a similar effect and are represented together, ●. **B,** The concentration dependence of flavonoid stimulation of germination in minimal medium is shown for naringenin, ▼ ; apigenin, ● ; and pisatin, ■. The percent germination was measured 9 h after inoculation of the medium. Inset: Percent germination represented for the lower concentrations of flavonoids. All results shown here are the average of three replicates and error bars represent standard errors of the mean. The results were confirmed by two independent experiments.

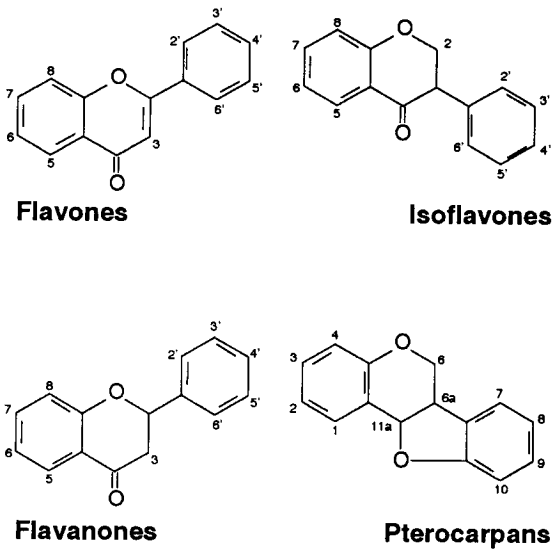

Fig. 2. Structures of flavonoids used. *Flavones:* apigenin (4′,5,7-trihydroxy-), apigenin-*O*-glucoside (4′,5-hydroxy-7-*O*-glucoside-), luteolin (3′,4′,5,7-tetrahydroxy-), 7-hydroxyflavone, 4′,7-dihydroxyflavone. *Flavonols (C-3 hydroxy flavones):* kaempferol (3,4′,5,7-tetrahydroxy-), fisetin (3,3′,4′,7-tetrahydroxy-flavone), quercetin (3,3′,4′,5,7-pentahydroxy-). *Isoflavones:* biochanin A (5′7-dihydroxy-4′-methoxy-), daidzein (4′,7-dihydroxy-), genistein (4′,5′7-trihydroxy-). *Flavanones:* hesperitin (3′,5,7-trihydroxy-4′-methoxy-), naringenin (4′,5,7-trihydroxy-), eriodictyol (3′,4′,5,7-tetrahydroxy-),. *Pterocarpans:* pisatin ((+) 6a-hydroxy-3-methoxy-8,8-methylenedioxy-), DMDP (3,6a-dihydroxy-8,9-methylenedioxy), medicarpin ((-)3 hydroxy, -9-methoxy-), maackiain (3-hydroxy-8,9-methylenedioxy-), phaseollin ((-) 3-hydroxy, fused-9,10 dimethylpyran ring-).

Barrios et al. 1993). This *nod* gene expression is necessary for initiating the symbiotic plant-bacterial interactions leading to nodulation. We describe here a characterization of flavonoid-responsive germination in *F. solani* f. sp. *pisi* which suggests that flavonoids in root exudates have the potential to initiate interactions with this pathogenic fungus as well. Further, the stimulatory action of the phytoalexin, normally inhibitory to many fungi and so thought to be part of a defense response, suggests that pathogens which have developed an ability to tolerate their inhibitory action may capitalize on these compounds for plant recognition as well.

RESULTS

Stimulation of macroconidia germination by nutrients.

F. solani f. sp. *pisi* macroconidia, the major spore type of the genus *Fusarium*, were used for germination experiments. Macroconidia of *F. solani* f. sp. *pisi* quickly germinated within 3 to 4 h in a rich medium (Barz medium). The spores displayed a much lower level of germination in a minimal medium (i.e., succinate medium) even after 15 h, as shown in Figure 1. No germination was observed in water alone. Supplementation of components from the rich medium into the minimal medium demonstrated that either casein hydrolysate (at 0.8% w/v) or sucrose (above 0.05%) produced a greater than 90% germination efficiency. Glucose (at 5%) produced 56% germination. Thus a mixture of amino acids or a fermentable carbon source seemed to provide a stimulus for germination. The succinate medium lacked the nutrients which provide this stimulus (germination of 0 to 20%) but did

Table 1. Flavonoid stimulation of spore germination of *Fusarium solani* pathogenic on pea or bean, and comparison to their *nod* gene induction in rhizobia specalizing on these hosts

	Spore germination % (±SE)[a] *Fusarium solani*		Activation of *Nod* genes in *Rhizobium leguminosarum*	
	f. sp. *pisi* (pea)	f. sp. *phaseoli* (bean)	bv. *viciae*[b] (pea)	bv. *phaseoli*[c] (bean)
Rich medium	100.0 (±0.0)	96.0 (±2.5)		
Minimal medium	10.3 (±5.0)	0.27 (±0.2)		
Flavanones				
Hesperitin	80.7 (±3.5)	35.0 (±0.0)	+++++	
Naringenin	72.0 (±3.8)	29.7 (±0.3)	++++	++
Eriodictyol	70.6 (±1.5)	6.0 (±0.9)	++++	+
Flavones				
Apigenin	79.0 (±5.5)	17.3 (±2.2)	+++++	
Apigenin-7-glucoside	64.8 (±3.6)	3.7 (±0.6)	++++	
Luteolin	60.3 (±3.9)	8.4 (±1.0)	++++	
7-hydroxyflavone	33.7 (±3.0)	1.3 (±0.4)	++	
4',7-dihydroxyflavone	26.8 (±4.7)	5.0 (±1.5)	++	
Flavonols				
Kaempferol	12.0 (±3.5)	8.2 (±1.1)	–	+
Fisetin	4.3 (±1.9)	3.0 (±1.8)	–	
Quercetin	4.3 (±1.9)	17.7 (±1.9)	–	+
Isoflavones				
Biochanin A	6.0 (±1.5)	83.7 (±2.3)		
Daidzein	11.3 (±2.6)	16.7 (±0.9)	–	
Genistein	18.0 (±5.3)	57.7 (±1.8)	–	++++
Pterocarpans				
Pisatin	75.7 (±5.6)	10.1 (±1.5)		
DMDP	79.3 (±4.6)	29.0 (±1.5)		
Medicarpin	78.0 (±3.1)	89.0 (±0.0)		
Maackiain	68.7 (±9.9)	92.3 (±0.3)		

[a] Germination percentages were determined after 9 h of incubation. Data are the averages of treaments each with three replicates with the standard error of the mean in parenthesis. Flavonoids were added to a final concentration of 50 μM in minimal medium.

[b] Data from Zaat et al. (1989) for the symbiont of pea. + indicates an approximate fivefold increase in β-galactosidase activity in *nodABC-lacZ* fusion at a 1 μM concentration of flavonoids; – indicates a lack of induction or a reduction in activity. Blanks indicate flavonoids not reported.

[c] Data for flavonols from bean seed (Hungria et al. 1989a) and others from bean root exudate (Hungria et al. 1989b) for the *nod* gene induction in the symbiont of bean. + indicates approximately 1,000 units of β-galactosidase activity in *nodABC-lacZ* fusion after addition of flavonoids at 10 μM, using the same strain in both studies. Blanks indicate flavonoids not reported.

support growth of this fungus once it has germinated (see below). Limited germination was also seen in minimal medium utilizing lactose as the carbon source. Limited germination has been reported for other *F. solani* strains with other minimal media containing different utilizable carbon sources or low glucose concentrations (Kwon and Epstein 1993; Schuerger et al. 1993).

Stimulation of germination by flavonoids.

In minimal medium, pisatin significantly increased both the proportion of macroconidia which germinate and the length of the germ tube arising from those that did germinate. A similar effect was observed with the related flavanone naringenin and flavone apigenin (Fig. 1A), which have been previously shown to induce *nod* gene expression in a range of rhizobia. The flavonoid concentrations required for inducing 50% and full germination were approximately 30 and 100 μM, respectively. The increase in germination compared to minimal medium alone was significant ($P \leq 0.001$, G-test) with the addition of 0.2 μM naringenin or with 1.0 μM pisatin or apigenin (Fig. 1B). Additional flavonoids were tested (Fig. 2) which display differential induction or inhibitory activity on *Rhizobium* species specializing on different hosts. The germination stimulating activity of these flavonoids are shown in Table 1. Germination of macroconidia was most strongly induced by the flavones apigenin, apigenin-7-*O*-glucoside, and luteolin, and flavanones naringenin, eriodictyol, and hesperitin, but not by flavanols or isoflavones. The effect of two highly stimulatory flavonoids was additive. For example, 5 μM apigenin-7-*O*-glucoside and eriodictyol individually produced 21% and 28% germination, respectively, but produced 48% germination when added together. The flavonoids displaying only weak stimulatory activity did not interfere with the action of strong stimulators (e.g., 50 μM kaemferol or pisatin individually produced 14% and 40% germination, respectively, but 61% germination when added together), and so their lack of stimulation does not appear to be due to a fungistatic property of those compounds. Chlamydospores, another type of spores involved in dispersal and survival of *F. solani* was also tested for flavonoid-induced germination. Pisatin, hesperitin, naringenin, luteolin, or apigenin treatment also increased the frequency of chlamydospore germination significantly (e.g., 50% germination with 50 μM apigenin, compared to 8% with DMSO alone). However, the effect was smaller and germination was more variable with chlamydospores than with macroconidia.

The stimulatory nature of pisatin was further studied by testing other legume pterocarpan isoflavonoids. Like pisatin (the phytoalexin produced by pea), medicarpin, and maackiain all significantly increased the percentage of germination of *F. solani* f. sp. *pisi* (Table 1). These phytoalexins are found in chickpea (another host of many *F. solani* f. sp. *pisi* strains) as well as a wide variety of other legumes (Ingham 1983). Phaseollin, one of several pterocarpan phytoalexins from

bean (*Phaseolus vulgaris*), produced little increase in germination at 100 μM (20% germination), but did increase germination at lower concentrations of 25 and 50 μM (80% and 69% germination, respectively). DMDP, the product of pisatin demethylation, stimulated germination as well as pisatin. Flavonoid-responsive stimulation did not appear to be due to utilization of these compounds as nutrients since addition of pisatin or other flavonoids to spores suspended in water alone did not stimulate germination (<2% germination with or without flavonoids). Also, the stimulation of germination by pisatin was similar in related strains which either contained or lacked pisatin demethylase (77-2-3 and 94-6-1, displaying 71% and 77% germination, respectively), the enzyme catalyzing the first step necessary for pisatin metabolism (VanEtten et al. 1989).

Flavonoid-responsive germination in other *Fusarium* spp.

To determine if flavonoid-responsive germination is shared by related fungi, other Fusaria were also tested. Macroconidia from *F. solani* f. sp. *phaseoli*, a pathogen of bean but not pea, were stimulated to germinate by the isoflavones genistein and biochanin A and the pterocarpans medicarpin and maackiain (Table 1). Partial stimulation was provided by the flavanones hesperitin and naringenin. Pisatin, apigenin, apigenin-7-*O*-glucoside, eriodictyol, and luteolin, which did stimulate *F. solani* f. sp. *pisi*, displayed only weak stimulation of *F. solani* f. sp. *phaseoli*. Phaseollin, tested at 50 μM only, did not stimulate germination of the bean pathogen. Two other soilborne Fusaria with nonleguminous hosts, *Fusarium acuminatum*, a pathogen commonly causing foot rot of graminaceous hosts, and *Nectria haematococca* MP I (anamorph *Fusarium solani*, strains associated with cucurbits) were also tested. Neither demonstrated any increase in germination with addition of any of the 11 flavonoids or pterocarpans tested (data not shown).

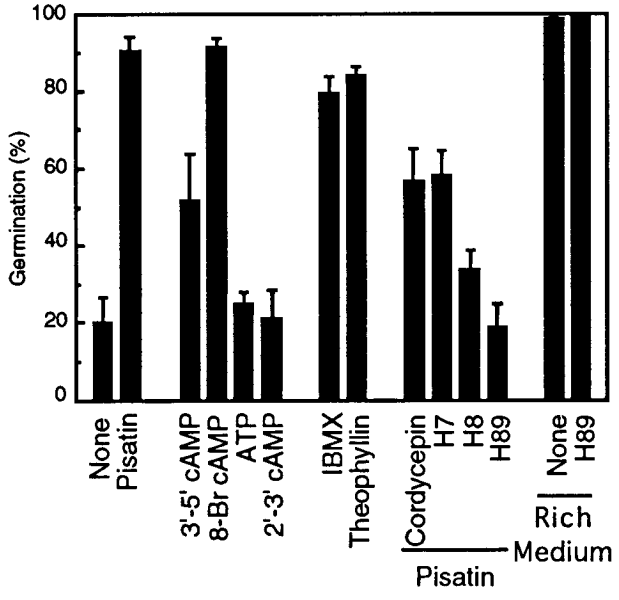

Fig. 3. Effect of cAMP analogues and inhibitors of the cAMP-dependent signal pathway upon germination. Macroconidia were suspended in minimal medium (except where rich medium noted) containing the supplements indicated. The percentage of macroconidia which germinated was measured at 9 h. The difference between no supplements and 50 μM pisatin provides a measure of flavonoid-stimulated germination. The exogenously added 3′-5′ cAMP (10 mM), or its analogue 8-br-cAMP (10 mM) was compared to a similar concentration of the inactive analogues ATP or 2′-3′ cAMP, all without the addition of flavonoid. The cAMP phosphodiesterase inhibitors IBMX (5 mM) and theophylline (10 mM) were also added without the addition of flavonoid. Cordycepin (10 mM), an inhibitor of adenylate cyclase, and H7, H8, or H89 (100 μM), inhibitors of cAMP-dependent protein kinase (PKA), were added along with 50 μM pisatin. Macroconidia suspended in rich medium (Barz) was compared without or with H89, the PKA inhibitor, shown on the far right. Values represent the mean for three replicates and error bars represent standard error of the mean. Results were confirmed by two independent experiments. Concentrations of the inhibitors were chosen from their effective concentrations reported previously for model systems.

Signal pathway leading to spore germination.

Because flavonoid stimulation of spore germination appears to involve specific recognition of and reaction to these compounds, we used a pharmacological approach to identify a potential signal pathway through which they stimulate germination. The action of cAMP, its analogs, and inhibitors of specific components in the cAMP-dependent signal transduction pathways are shown in Figure 3. Exogenous addition of (3′-5′) cAMP increased germination to approximately half that seen by the addition of flavonoids. Addition of 8-bromo-cAMP, an active analogue which is more lipophilic than cAMP, produced greater stimulation, equivalent to that of the most active flavonoid. Both 2′-3′ cAMP and ATP, inactive analogues of 3′-5′ cAMP in model systems, failed to stimulate germination. Addition of phosphodiesterase inhibitors IBMX and theophylline, which increase the intracellular concentration of cAMP by reducing its turnover, each increased germination in the absence of flavonoids. To determine if flavonoid-responsive germination specifically utilized components of the cAMP pathway, inhibitors of enzymes within this pathway were tested for their ability to interfere with the stimulatory activity of the flavonoids. Cordycepin, an inhibitor of adenylate cyclase (Chiba et al. 1992), and inhibitors of cAMP-dependent protein kinase (H7, H8, and H89) reduced or prevented the ability of pisatin to stimulate germination (Fig. 3). The level of inhibition displayed by the three kinase inhibitors (H89 > H8 > H7) paralleled the relative specificity and strength of these inhibitors towards cAMP-dependent protein kinase A (PKA) in model systems (Chijiwa et al. 1990; Hidaka et al. 1984). The strongest and most specific inhibitor, H89, also limited the germination of macroconidia in response to other flavonoids tested, including apigenin and naringenin (data not shown).

In contrast to the above results, germination in rich medium was not inhibited by H89 (Fig. 3), H7, H8, or cordycepin. Similar insensitivity to these inhibitors was observed with nutrient-stimulated germination resulting from supplementing minimal medium with only casein hydrolysate or sucrose (>90% germination with or without H89 treatment). Under certain circumstances of partial germination in rich medium, the germination rate was increased slightly by the addition of H89. This increase was not observed in water. Together, these results indicate that flavonoid-responsive germination utilizes a signal pathway which is sensitive to inhibition by a limitation of PKA activity or cAMP levels, whereas nutrient-responsive germination utilizes a pathway which is not inhibited by changes in the activity of these components.

Effect of cAMP on induction of *PDA*1 promoter expression.

Pisatin induces both germination and expression of the pisatin demethylase (*PDA*1) gene responsible for detoxification of pisatin. The possibility for a shared signal transduction pathway leading to both responses was tested. A stable *N. haematococca* MPVI transformant (strain 94-6-1) carrying a *PDA*1::*GUS*A (β-glucuronidase) transcriptional fusion was used to measure the amount of *PDA*1 promoter stimulation. Germination of this strain was induced by treatment of macroconidia with pisatin, 8 Br-cAMP, or IBMX in minimal medium. β-Glucuronidase activity was determined in germlings 9 h after treatment by staining with X-Glu substrate, which produces an insoluble blue product due to GUS activity (Jefferson 1987). The germlings resulting from pisatin treatment of macroconidia stained dark blue, indicating strong induction of *PDA*1 promoter expression. In contrast, the germlings resulting from treatment of macroconidia with 8 Br-cAMP or IBMX remained unstained with X-Glu, indicating the lack of *PDA*1 promoter induction. These results indicate that increasing extracellular or intracellular cAMP levels alone does not mimic pisatin's stimulation of *PDA*1 expression, unlike that found for pisatin's stimulation of germination.

Stimulation of macroconidia germination in root exudates.

The ability of the PKA inhibitors to inhibit flavonoid-induced germination but not nutrient-induced germination provided a tool to test the relative importance of these signals in root exudates. Germination of spores in the presence of pea root with and without the PKA inhibitor is shown in Figure 4. Macroconidia incubated in the presence of a single pea root in water germinated at a much greater rate than those in water alone. The stimulatory effect was evident both in suspended conidia (Fig. 4A) and also macroconidia attached to the root. When the PKA inhibitor, H89, was added to the spore suspension just prior to the root, subsequent germination was greatly reduced. This was true for macroconidia both remaining suspended (Fig. 4A) and attached to the root surface (not shown). Although independent batches of pea seedlings displayed differing levels of exudate-stimulated expression, the amount of H89-dependent decrease was similar in each. These results indicate that a major stimulatory factor in pea root exudates has similar PKA inhibitor-sensitivity as that of flavonoids tested in culture, and is in contrast to the insensitivity of nutrient-induced germination. The results with pea root were compared to corn root since corn does not produce flavonoid biosynthetic enzymes in the root (Rommeswinkel et al. 1992). *F. solani* f. sp. *pisi* spores displayed only a small increase in germination when corn root was present, and this was not reduced when H89 was included. Pea-induced germination was not only observed in the presence of the root but also with cell-free pea seedling exudates alone. A water rinsate of pea seedlings provided substantial stimulation of germination which was sensitive to H89, like that in response to pea root and flavonoids (Fig. 4B).

The nature of the stimulus in pea rinsate was explored by determining if certain treatments would remove the stimulatory activity. Flavonoids are relatively nonpolar and are extractable from aqueous solutions with organic solvents. Extraction of the pea rinsate with hexane yielded stimulatory activity in the hexane fraction (Fig. 4B). The stimulatory activity was also removed from the pea rinsate by passage over a C18 Sep-Pac column which binds nonpolar compounds (1.2% germination in the column flow through). Stimulatory activity was eluted from the C18 column with organic solvent

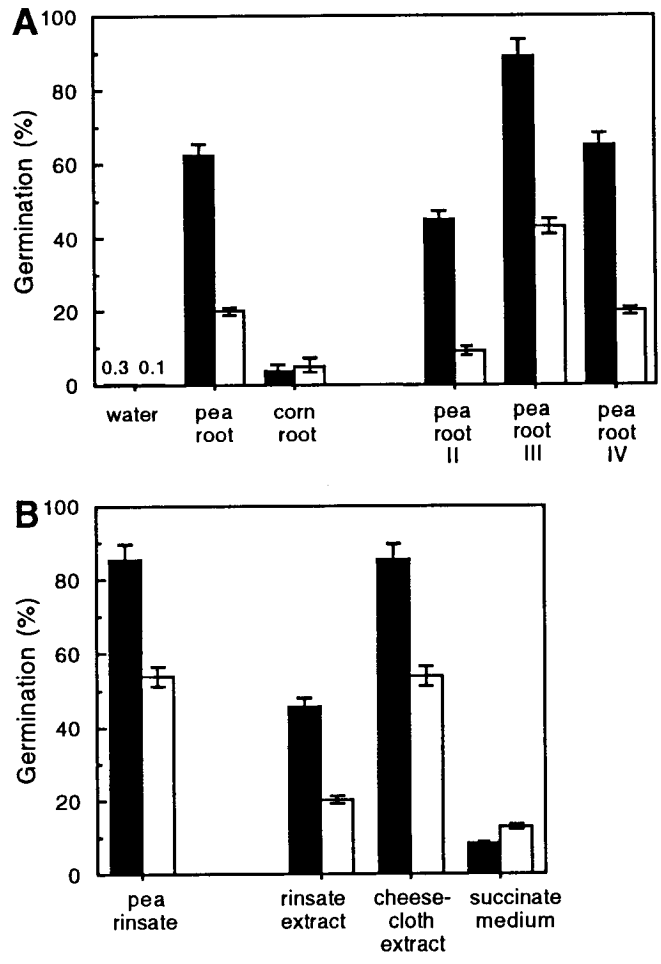

Fig. 4. Germination of *F. solani* f. sp. *pisi* macroconidia in the presence of root exudates of pea and corn, and partition of stimulatory activity in pea rinsates. Macroconidia were suspended without (dark bars) or with 100 μM H89 (light bars), an inhibitor of PKA. Percent of spores which had germinated after 6 h of gentle agitation is shown for each treatment. **A,** Spores were incubated in water alone (water) or in the presence of the root of an intact 6-day-old pea seedling (pea root) or corn seedling (corn root) in 200 μl of water. Germination was scored for macroconidia which remained suspended. Values indicate mean of three replicates and error bars indicate the standard error of the mean. Independent replicates of germination in the presence of pea root are shown, using different preparations of pea seedlings (pea root II, III, and IV); statistical evaluation of the percent germination by the G-test indicates a significant reduction in germination by H89 ($P < 0.01$) in each replicate. **B,** Germination in plant-free preparations. A water rinsate of pea seedlings (pea rinsate) was made from 8-day-old seedlings with the cotyledon removed. Spores were added to 400 μl of rinsate which was used without concentration. A hexane extract of this pea rinsate (rinsate extract) was made and was added to spores in 0.1× succinate medium in levels equivalent to the concentration in the original rinsate. Cheesecloth on which pea seedlings were grown was extracted with hexane (cheesecloth extract) and was added to spores in 0.1× succinate medium. Before use, both organic solvent extracts above were evaporated to dryness, resuspended in dimethylsulfoxide (DMSO), and added to spores suspended in 400 μl of 0.1× succinate medium. Germination in 0.1× succinate medium alone is shown (succinate medium).

washes which remove flavonoids from the column (98% germination from a 55:45 methanol/water elution). Flavonoids and other phenolic compounds are absorbed by polyvinylpolypyrrolidone (PVPP) (Doner et al. 1993). Treatment of the pea rinsate with insoluble PVPP reduced its ability to stimulate germination (34.8% germination in PVPP-treated rinsate compared to 94.8% in untreated rinsate). PVPP treatment of rich medium did not reduce its stimulatory activity. Stimulatory activity was also found to be present in a hexane extract of the cheesecloth on which the pea seedlings were grown. This stimulatory activity was sensitive H89 inhibition (Fig. 4B). These results indicate that a significant portion of the stimulatory activity in pea exudates appears to have general physical properties in common with flavonoids.

DISCUSSION

The stimulation of spore germination by plant root exudates is a widely observed phenomenon and is a critical step for initiating plant-fungal interactions with many soilborne pathogens. However, in only a few cases have plant-derived compounds beyond sugars or amino acids been implicated in this signaling. A notable example of a host-specific signal is the ability of volatile alkyl sulphides, ultimately derived from host *Allium* species, to induce germination of *Sclerotium cepivorum* sclerotia (Coley-Smith and King 1970). The apparent lack of more specific signals among other fungal pathogen systems contrasts with that of plant-pathogenic plants and symbiotic and pathogenic bacteria, where host phenolics and flavonoids stimulate the interaction with the host (Lynn and Chang 1990). The present study indicates that *F. solani* strains pathogenic on two different legumes can utilize certain plant flavonoids as a stimulus for germination of macroconidia. The results indicate that this flavonoid response appears to be specific and not merely due to utilization of these compounds as a nutritional source. The stimulatory action of the flavonoids is intriguing in suggesting that these compounds may provide a more host-specific stimulatory signal in legume root exudates than nutrients, and could be utilized for microbial recognition of the plant for initiating both pathogenic and symbiotic interactions with legumes.

Examination of the flavonoid specificity for induction of *F. solani* f. sp. *pisi* spore germination indicates that C-4′ hydroxy- or methoxy-, C-7 and C-5 hydroxy-, C-3 deoxy- flavones or flavanones produced the strongest response. This specificity is very similar to that observed for *nod* gene induction in *R. leguminosarum* bv. *viciae*, the symbiont of pea (Table 1). The similarity is striking because different species or biovars of rhizobial bacteria, which specialize on other legumes, generally display different flavonoid specificities in *nod* gene induction (Hungria et al. 1991b; Kosslak et al. 1987; Peters and Long 1988; Zaat et al. 1989). The major nod-inducing flavonoids exuded by pea root have been reported to be apigenin-7-*O*-glucoside and eriodictyol (Firmin et al. 1986), based on HPLC analysis. Both of these compounds were highly stimulatory to germination of the *F. solani* pathogenic on pea. The parallel specificity between this pea pathogen and the pea rhizobia, among a number of flavonoids tested, is significant in suggesting that other flavonoids which are released by pea and induce nodulation by this bacteria would also stimulate spore germination of *F. solani* f. sp. *pisi*.

The related bean pathogen, *F. solani* f. sp. *phaseoli*, displayed a similar flavonoid response, but with a different pattern of specificity. Its germination was induced by the isoflavan genistein and the flavanone naringenin. Both compounds have been shown to be inducers of *nod* genes in the bean symbiont, *R. leguminosarum* bv. *phaseoli* (Table 1), and both have been found to be major flavonoids present in bean root exudates (Hungria et al. 1991b). Therefore it appears that *Fusarium solani* strains specializing on different legumes may have developed an ability to respond to flavonoids released by their respective host. The overlapping spectra of flavonoids produced by some legumes (e.g., eriodictyol has been identified in exudate from both pea and bean, Firmin et al. 1986; Hungria et al. 1991b) makes it is unlikely that such differences in flavonoid specificity between the two *F. solani* formae speciales would establish strictly host-selective germination by itself. However, the lack of flavonoid-responsive germination in the two *Fusarium* species pathogenic on non-leguminous hosts suggests that flavonoids could provide a certain level of host-specific recognition among these legume pathogens.

Although it is not possible to directly evaluate the importance of flavonoid-responsive germination in the rhizosphere based on our results, its contribution in root exudates appears to be potentially significant. The concentration required for stimulating germination is compatible with the levels of flavonoid exudation reported for legume roots. For example, a healthy bean seedling releases 337 nmoles of naringenin, 281 nmoles of eriodictyol, and 42 nmoles of genistein per day per plant (Hungria et al. 1991b). This level of genistein or naringenin exudation would individually induce significant germination of *F. solani* f. sp. *phaseoli*, the bean pathogen, if dispersed in a volume of 1 ml. Although the flavonoid levels have not been measured in pea exudate, a level of eriodictyol or other flavonoids similar to that in bean exudate would maximally induce germination of *F. solani* f. sp. *pisi* even if distributed over a volume of 10 ml. The overall effective concentration may be greater since legume root exudates often contain multiple *nod*-inducing flavonoids and the stimulatory effect of multiple flavonoids on germination was found to be additive. Additionally, rhizobial infection has been shown to increase the level or number of *nod*-stimulating flavonoids released in several legumes (Dakora et al. 1993; Recourt et al. 1991).

Our in vivo results indicate that healthy pea roots do appear to release stimulators of spore germination. The ability of the PKA inhibitors to inhibit flavonoid-induced germination but not nutrient-induced germination provided a tool to test the relative importance of these signals in root exudates. Since a major proportion of germination in the presence of pea root or in seedling rinsate is sensitive to such inhibitor, nutrients (i.e., amino acids and sugars) are unlikely to be the sole stimulants in root exudates. Further, the stimulus in the rinsate has properties similar to flavonoids. It is extractable with hexane and binds to hydrophobic C18 columns, indicating a relatively nonpolar character like the flavonoids. The stimulus was also removed from the pea rinsate with PVPP, a highly hydrophilic polymer that binds flavonoids, as well as other phenolics, through hydrogen bonding with their hydroxyl groups (Doner et al. 1993). These results are consistent with a supposition that flavonoids are responsible for a significant proportion of the increased germination in root exudate. Although it is

possible that other stimulatory compounds with similar properties are also present in the pea root exudate and also act through a PKA-dependent pathway, such stimulatory factors appear to be absent in corn root exudate. Corn roots have been found to lack expression of key flavonoid biosynthetic enzymes or *nod* gene-inducing activity (Firmin et al. 1986; Rommeswinkel et al. 1992) and so would not be expected to release flavonoids in root exudate. Thus correlative evidence using the PKA inhibitor and variations in both the plant and fungal pathogens point towards a potentially significant role for flavonoids as a stimulatory factor in pea root exudate.

The stimulatory activity of the pterocarpan phytoalexins on *F. solani* f. sp. *pisi* is surprising since they generally inhibit germination of spores and hyphal growth of many fungi at similar concentrations in rich medium (Smith 1982). This fungistatic activity, as well as further experimental evidence, has indicated a potential role for these compounds in host defense (VanEtten et al. 1989). The ability of the pterocarpan phytoalexins to stimulate germination appears to be due to chemical recognition, rather than from their fungistatic activity. DMDP, the product of pisatin demethylase, lacks the toxicity of pisatin (VanEtten et al. 1975) but stimulated germination as well as pisatin. Also, addition of micromolar concentrations of H_2O_2 or Cu^{++}, which may mimic the fungistatic action of the phytoalexins, did not induce germination. The stimulatory activity is further surprising since pterocarpan phytoalexins have been found to not induce *nod* genes expression in the rhizobial systems tested (Dakora et al. 1993). Thus the pterocarpans represent an extension of the range of stimulatory flavonoids beyond those found to induce rhizobial nodulation. Because plants produce phytoalexins in response to environmental stress or microbial challenge, these compounds may provide an additional strong stimulus from roots experiencing such stresses under natural conditions. Pterocarpan phytoalexins have been shown to be exuded from legume roots and so are thought to influence microbes in the rhizosphere (Phillips and Kapulnik 1995). Exudates from soybean and alfalfa roots inoculated with rhizobial symbionts or pathogens contain the pterocarpan phytoalexins glyceollin and medicarpin, respectively (Dakora et al. 1993; Schmidt et al. 1992). Glyceollin levels reached as high as 48 nmoles/plant within 20 h of inoculation. Pisatin accumulates in pea to concentrations as high as 5 mg/cm^3 (at least 15 mM) in diseased epidermal tissue (Pueppke and VanEtten 1974), and so may be similarly exuded from pea roots to provide a stimulus for pathogen germination. Use of the pterocarpan phytoalexins as a stimulus would probably require a certain level of tolerance to these compounds. This requirement was evident with *F. solani* f. sp. *pisi*. This fungus possesses degradative tolerance to pisatin, medicarpin, and maackiain (Lucy et al. 1988; VanEtten et al. 1989), and its germination was induced with 100 μM of these compounds. In contrast it was not stimulated by similar concentration of phaseollin, the bean phytoalexin, to which its hyphal growth is more sensitive (VanEtten and Stein 1978). The stimulatory action of the phytoalexins is intriguing in suggesting that pathogens may have developed mechanisms not only to tolerate the inhibitory action of host isoflavonoid phytoalexins but also to capitalize on these compounds for plant recognition as well.

Cyclic AMP (cAMP) levels or the activity of a cAMP-dependent protein kinase (PKA) have been associated with the developmental processes in other filamentous fungi. These processes include dimorphic growth in *Ustilago maydis* and production of appressoria in *Magnaporthe grisea*, both in response to environmental cues (Gold et al. 1994; Lee and Dean 1993). Similar components appear to act in the signal transduction pathway leading to spore germination in *F. solani*. Increasing the intracellular level of cAMP induced germination and inhibitors of adenylate cyclase or PKA prevented germination. It was significant, however, that these inhibitors interfered with flavonoid-stimulated germination but not with nutrient-stimulated germination. This differential inhibition is unlikely to be due to differences in the uptake of the inhibitor in the two media since H89 treatment inhibits hyphal elongation at later stages of hyphal growth in either medium, an effect consistent with the requirement for PKA function in hyphal growth of *Neurospora crassa* (Yarden et al. 1992). The differential action of the inhibitors upon germination in *F. solani* suggests that flavonoid-responsive germination may be mediated through cAMP or PKA but that nutrient-responsive germination acts through a separate signal pathway, as shown in Figure 5. A role for PKA in response to one environmental stimulus and not another would differ from the other PKA-dependent developmental pathways in filamentous fungi described above, but has been suggested in germination of the slime mold *Dictyostelium* (Lydan et al. 1994). Flavonoids and nutrients may utilize separate signal pathways which regulate common genes. Glucose-responsive growth control in *Saccharomyces cerevisiae* is mediated by separate RAS/PKA-dependent and MAP kinase-dependent pathways (Costigan and Snyder 1994). A similar PKA-dependent pathway does not appear to mediate glucose responses in other fungi, such as *Neurospora crassa* or *Schizosaccharomyces pombe* (Thevelein 1991) and so could be recruited for niche-specific signals. Alternatively, flavonoids may stimulate germination by inducing the utilization of endogenous resources in the spore, such as trehalose. Trehalase activity is activated through phosphorylation by PKA in *S. cerevisiae* (Pernambuco and Thevelein 1994). Similar action in *F. solani* in response to flavonoid induction of PKA could

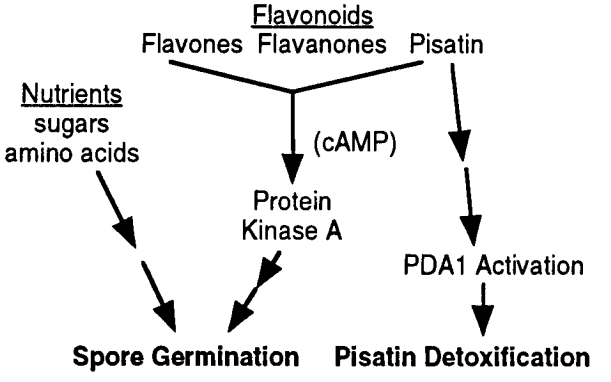

Fig. 5. Proposed partition of signal pathways leading to germination. Nutrient-stimulated germination of macroconidia is through a PKA-independent pathway, in contrast to the PKA-dependent stimulation produced by the flavonoids (flavones, flavanones, and pterocarpan isoflavonoids including pisatin). Pisatin stimulation of pisatin demethylase gene expression in *Nectria haematococca* appears to be through an independent pathway, since it is not induced by exogenous cAMP and has high inducer specificity for pisatin and DMDP alone.

provide endogenous nutritional signals for germination. The inhibitor results in this study indicate specific targets which allow testing of these possible mechanisms through molecular genetics.

The signal pathway leading to flavonoid-responsive germination also appears to differ from a second flavonoid-induced response in *F. solani* f. sp. *pisi*, that of *PDA*1 gene expression to produce pisatin detoxification activity (Fig. 5). Germination was induced by a much broader range of flavonoids than induce expression of *PDA*1 (pisatin and DMDP alone, VanEtten and Barz 1981). Also, treatments which increased intracellular cAMP levels induced germination but not *PDA*1 expression. The presence of multiple flavonoid responses which are not coregulated is similar to that observed with rhizobial bacteria where growth induction and induced tolerance to a host phytoalexin (glyceollin) are independent of the *nodD* gene required for induction of *nod* gene expression (Hartwig et al. 1991; Parniske et al. 1991).

Since fungi utilize a variety of biological niches, different fungi may respond to different stimuli for properly timing germination. For example germination of the avocado fruit pathogen, *Colletotrichum gloeosporioides*, is induced by waxes present on host fruit (Podila et al. 1993). *Sclerotium cepivorum* sclerotia germinate in response to volatile host-derived alkyl sulphides (Coley-Smith and King 1970). Flavonoids could provide a potential signal for soilborne fungi which interact with legume roots. Although flavones and flavanones occur in a variety of organs in many plants, their biosynthesis and exudation from roots has been best characterized in legumes (Rommeswinkel et al. 1992; Hungria et al. 1991b; Kosslak et al. 1987; Zaat et al. 1989). Isoflavonoids, such as the pterocarpans, would provide an even more unique signal, being principally found in the legumes (Ingham 1983). Because flavonoid release is required for initiating the interaction with most symbiotic nitrogen-fixing rhizobia, legumes would not be likely to lose this trait. Therefore it is possible that flavonoid-responsive germination is present in additional soilborne fungal pathogens of legumes. This is supported by different flavonoid responses in lower fungi which also interact with legumes. For example, flavonoids stimulate zygospore germination of vesicular-arbiscular (VA) mycorrhizal *Gigaspora* and *Glomus* strains which interact with legumes (Gianinazzi-Pearson et al. 1989; Tsai and Phillips 1991). These zygomycetes form obligate symbiotic endomycorrhizzal relationships with the host and this response has been used to attempt culturing of these fungi. In a second case, the isoflavones daidzein and genistein were shown to be chemoattractants for the motile zoospores of *Phytophthora sojae*, a pathogen of soybean (Morris and Ward 1992). Our findings with *Fusarium solani* are significant in identifying flavonoid-responsive development in the higher fungi as well. The use of kinase inhibitors presented here indicates a potential signal pathway through which such flavonoid responses may be mediated. It also provides initial experimental evidence that flavonoids in root exudates may be one component that initiates a fungal response. Unlike the mycorrhizal germination and zoospore chemotaxis, the *F. solani* germination response is extended beyond the *nod* gene-inducing flavonoids to include pterocarpan phytoalexins. Recognition of phytoalexins could be part of a similar fungal response in other pathogenic interactions as well.

F. solani f. sp. *pisi* offers a unique experimental system for studying flavonoid-responsive development, providing tools for further genetic manipulation of this trait through sexual genetics, having an easily cultured haploid vegetative state, and possessing a second flavonoid-response which is characterized at the level of gene expression. We have recently observed sufficient variation in flavonoid specificity and response in other *N. haematococca* MPVI strains isolated from plants other than pea to allow genetic analysis of flavonoid specificity and the role of this flavonoid response in the early events of the plant-microbe interaction.

METHODS

Fungal strains.

F. solani f. sp. *pisi* strain 77-2-3 (Kistler and VanEtten 1984) was used unless otherwise noted. *F. solani* f. sp. *phaseoli* strain 22678, and *N. haematococca* MP I strain T334 were kindly provided by H. D. VanEtten, University of Arizona. *Fusarium acuminatum* was provided by G. Goth, USDA/ARS, Beltsville, MD. *Fusarium* strains were maintained on V8 agar slant cultures grown at 20°C to promote formation of macroconidia. The *PDA*1::*GUSA* transcriptional fusion was present in a stable transformant (Tx13B4) of *F. solani* f. sp. *pisi* strain 94-6-1. The *PDA*1::*GUSA* contained *PDA*1 sequences −1187 to +3, relative to the translational start site and was introduced into *N. haematococca* using standard techniques (Stahl and Schäfer 1992). Transformants bearing this construct display the same pisatin inducibility in mature mycelia as the native *PDA*1 gene (unpublished).

Flavonoids and inhibitors.

Flavonoids were obtained from the following commercial sources: apigenin, biochanin A, 7-hydroxyflavone, and naringenin were purchased from Aldrich (Milwaukee, WI); apigenin-7-*O*-glucoside, daidzein, 7-4′-dihydroxyflavone, eriodictyol, fisetin, genistein, hesperitin, kaempferol, and luteolin from Indofine (Somerville, NJ); quercetin from Sigma (St. Louis, MO). Isoflavonoid phytoalexins were from noncommercial sources and were purified by repeated thin-layer chromatography. Pisatin was isolated from peas and purified by published procedures (VanEtten and Barz 1981). DMDP was prepared from addition of pisatin to a culture of a *Aspergillus nidulans* transformant containing the *PDA*1 gene (Schäfer et al. 1989) and subsequent purification of DMDP by preparative TLC. Medicarpin and maackiain were gifts from Vivian Miao. All flavonoid compounds were dissolved in DMSO at a stock concentration of 25 mM.

The 3′-5′ cAMP, 8-bromo-cAMP, 2′-3′ cAMP, 3′ deoxyadenosine (cordycepin), ATP, and theophylline(1,3-dimethylxanthine) were obtained from Sigma. H7[1-(5-isoquinolinesulfonyl)-2-methylpiperazine], H8{N-[2-(methylamino)-ethyl]-5-isoquinolinesulfonamide}, H89{N-[2-(3-(4-bromopheny)-2-propeny)-amino-ethyl]-5-isoquinoline} and IBMX (3-isobuty-1-methylxanthine) were from Calbiochem (San Diego, CA). IBMX, 8-bromo-cAMP, and theophylline were dissolved in DMSO as stock solution. Others were dissolved in water.

Germination.

Macroconidia were collected by washing mycelia growing on V8 agar slant cultures that were 10 to 20 days old. The

conidia were concentrated by centrifugation and resuspended in water, and the concentration was estimated with a hemacytometer. This suspension was used to inoculate 0.4 ml of growth medium to a final concentration of 10^6 conidia per ml. Succinate medium contains 170 mM succinate, 19 mM NH_4NO_3 and mineral salts (VanEtten and Barz 1981). Barz medium contains 277 mM glucose, 0.8% (w/v) casein hydrolysate (approximately 72 mM amino nitrogen), 0.05% (w/v) yeast extract, and mineral salts (VanEtten and Barz 1981). Less background germination was obtained in 0.1× succinate medium without diminishing flavonoid stimulation. Spore suspensions, including test compounds added in DMSO or water, were incubated in 12-ml culture tubes or 12-well culture plates at 24°C with shaking at 100 rpm to keep the spores suspended. DMSO alone was added to control reactions in minimal medium without flavonoid treatment. At the end of incubation, Tween 20 was added to 2% (v/v) followed by vigorous vortexing or pipetting to break any aggregation of germination tubes. Samples were examined under a compound microscope and 200 to 300 total spores were evaluated to determine the germination percentage. Experiments included three replicates of each treatment and results were confirmed in three additional experiments. X-Glu (5-bromo-4-chloro-3-indoyl-glucuronide) staining was performed by the methods of Jefferson (Jefferson 1987) in 100 µl of reaction buffer with no fixation.

Chlamydospores were produced from macroconidia in cultures of *F. solani* f. sp. *pisi* grown on V8 agar for 6 mo. Germination was performed at a concentration of 1×10^6 chlamydospores per ml in water with or without 50 µM flavonoids.

Plant exudates.

Pisum sativum (L.) cv. Alaska-2B seeds (Burpee Seed Co.) were surface sterilized by immersion in 0.25% sodium hypochlorite, 70% ethanol for 15 min and rinsed with sterile water. IO Cheif corn seed was surface sterilized in 0.25% sodium hypochlorite for 5 min and rinsed as above. Seeds were germinated on four layers of cheesecloth wetted with 0.1× Hoagland's solution in sterile boxes grown in darkness. Tests in the presence of roots were performed with seedlings which were 6 days old; these were gently removed and then inserted into sterile tubes containing sterile water containing the stated amendments such that the tip of the tap root was submerged. After incubation for 6 h with gentle agitation, the percentage of germinated macroconidia was measured for 250 macroconidia. Roots were stained with cotton blue/lactophenol. Root exudate from pea seedlings was prepared from 8-day-old pea seedlings. The cotyledon was carefully removed from the seedlings and the seedlings were soaked for 30 min in 500 ml of sterile distilled water. The rinsate was filtered through a GF/A glass fiber filter (Whatman, nominal exclusion of 1.6 µm) and used without further concentration. A 50-ml sample of this rinsate was extracted twice with an equal volume of hexane. The hexane layer was evaporated to dryness in a rotovap and resuspended in 0.5 ml of DMSO. Four microliters of this extract was added to 400-µl volume to test germination. Preliminary solid-phase separation was performed with C18 Sep-Pac cartridges (Waters, Milford, MA) using 25 ml of pea rinsate. PVPP treatment of the rinsate involved addition of insoluble polyvinylpolypyrrolidone (P-6755, Sigma Chemical Co., St Louis, MO) to the rinsate (8%, w/v) and shaking at 16°C for 3 h. The PVPP was removed by centrifugation and this treatment was repeated once with the supernatant. The cheesecloth used to grow the pea seedlings was extracted with an equal volume of hexane for 3 h. The hexane layer was separated from the aqueous layer and evaporated to dryness in a rotovap and was resuspended in 13 ml of ethanol. Two milliliters of this extract was dried, resuspended in 0.4 ml DMSO, and 2 µl was added to the 400 µl volume used in germination experiments.

ACKNOWLEDGMENTS

We wish to thank H. VanEtten, G. Bean, and G. Goth for supplying strains. This research was supported by grants from the National Agricultural Research Project and the National Science Foundation (MCB-9419266). Maryland Agriculture Experiment Station Article #A6664.

LITERATURE CITED

Chijiwa, T., Mishima, A., Angiwara, M., Sano, M., Hayoushi, K., Inoue, T., Naito, K., Toshika, T., and Hidaka, H. 1990. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) of PC12D pheochromocytoma cells. J. Biol. Chem. 265:5267-5272.

Coley-Smith, J. R., and King, J. E. 1970. Response of resting structures of root infecting fungi to host root exudates: An example of specificity. Pages 130-133 in: Root Diseases and Soil-borne Pathogens T. A. Toussoun, R. V. Bega, and P. E. Nelson, eds. University of California Press, Berkeley.

Cook, R. J., and Snyder, W. C. 1965. Influence of host exudates on growth and survival of germlings of *Fusarium solani* f. *phaseoli* in soil. Phytopathology 55:1021-1025.

Costigan, C., and Snyder, M. 1994. SLK1, a yeast homolog of MAP kinase activators, has a RAS/cAMP-independent role in nutritional sensing. Mol. Gen. Genet. 243:286-296.

Dakora, F. D., Joseph, C. M., and Phillips, D. A. 1993. Alfalfa (*Medicago sativa* L.) root exudates contain isoflavonoids in the presence of *Rhizobium meliloti*. Plant Physiol. 101:819-824.

Doner, L. W., Becard, G., and Irwin, P. L. 1993. Binding of flavonoids by polyvinylpolypyrrolidone. J. Agric. Food Chem. 41:753-757.

Firmin, J. L., Wilson, K. E., Rossen, L., and Johnston, A. W. B. 1986. Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. Nature 324:90-92.

Fisher, R. F., and Long, S. R. 1992. *Rhizobium*-plant signal exchange. Nature 357:655-660.

Gianinazzi-Pearson, V., Branzanti, B., and Gianinazzi, S. 1989. In vitro enhancementof spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7:243-255.

Gold, S., Duncan, G., Barrett, K., and Kronstad, J. 1994. cAMP regulates morphogenesis in the fungal pathogen *Ustilago maydis*. Genes Dev. 8:2805-2816.

Hartwig, U. A., Joseph, C. M., and Phillips, D. A. 1991. Flavonoids released naturally from alfalfa seeds enhance growth rate of *Rhizobium meliloti*. Plant Physiol. 95:797-803.

Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. 1984. Isoquinoline sulfonmmides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23:5036-5041.

Hungria, M., Joseph, C. M., and Phillips, D. A. 1991a. Anthocyanidins and flavonols, major nod gene inducers from seeds of a black-seeded common bean (*Phaseolus vulgaris* L.). Plant Physiol. 97:751-758.

Hungria, M., Joseph, C. M., and Phillips, D. A. 1991b. *Rhizobium nod* gene inducers exuded naturally from roots of common bean (*Phaseolus vulgaris* L.). Plant Physiol. 97:759-764.

Ingham, J. L. 1983. Naturally occurring isoflavonoids (1855-1981). Pages 1-266 in: Progress in the Chemistry of Organic Natural Prod-

ucts. Vol. 43. W. Herz, H. Grisebach, and G. W. Kirby, eds. Springer-Verlag, Wein.
Jefferson, R. A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5:387-405.
Kistler, H. C., and VanEtten, H. D. 1984. Regulation of pisatin demethylation in *Nectria haematococca* and its influence on pisatin tolerance and virulence. J. Gen. Microbiol. 130:2605-2613.
Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H. E., and Appelbaum, E. R. 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. Proc. Natl. Acad. Sci. USA 84:7428-7432.
Kwon, Y. H., and Epstein, L. 1993. A 90-kDa glycoprotein associated with adhesion of *Nectria haematococca* macroconidia to substrata. Mol. Plant-Microbe Interact. 6:481-487.
Lee, Y. H., and Dean, R. A. 1993. cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. Plant Cell 5:693-700.
Leon-Barrios, M., Dakora, F. D., Joseph, C. M., and Phillips, D. A. 1993. Isolation of *Rhizobium melloti nod* gene inducers from alfalfa rhizosphere soil. Appl. Environ. Microbiol. 59:636-639.
Lucy, M. C., Matthews, P. S., and VanEtten, H. D. 1988. Metabolic detoxification of the phytoalexins maackiain and medicarpan by *Nectria haematococca* field isolates: Relationship to virulence on chickpea. Physiol. Mol. Plant Pathol. 33:187-199.
Lydan, M. A., Cotter, D. A., and O'Day, D. H. 1994. Stage-specific changes in protein phosphorylation during spore germination in *Dictyostelium*: Role of calmodulin. Biochem. Biophys. Res. Comm. 201:430-435.
Lynn, D. G., and Chang, M. 1990. Phenolic signals in cohabitation: Implications for plant development. Annu. Rev. Plant Physiol. 41:497-526.
Morris, P. F., and Ward, E. W. B. 1992. Chemoattraction of zoospores of the soybean pathogen, *Phytophthora sojae*, by isoflavones. Physiol. Mol. Plant Pathol. 40:17-22.
Nelson, E. B. 1991. Exudate molecules initiating fungal responses to seeds and roots. Pages 197-209 in: The Rhizoshere and Plant Growth. L. Keister and P. B. Cregan, eds. Kluwer, Amsterdam.
Nelson, E. B., and Hsu, J. S. 1994. Nutritional factors affecting responses of sporangia of *Pythium ultimum* to germination stimulants. Phytopathology 84:677-683.
Parniske, M., Ahlborn, B., and Werner, D. 1991. Isoflavonoid-inducible resistance to the phytoalexin glyceollin in soybean rhizobia. J. Bacteriol. 173:3432-3439.
Pernambuco, M. B., and Thevelein, J. M. 1994. Activation of trehalase during growth induction by nitrogen sources in the yeast *Saccharomyces cerevisiae* depends on the free catalytic subunits of CAMP-dependent protein kinase, but not on functional Ras proteins. Yeast 10:1049-1064.
Peters, N. K., and Long, S. R. 1988. Alfalfa root exudates and compounds which promote or inhibit induction of *Rhizobium meliloti* nodulation genes. Plant Physiol. 88:396-400.
Phillips, D. A., and Kapulnik, Y. 1995. Plant isoflavonoids, pathogens and symbionts. Trends Microbiol. 3:58-63.
Podila, G. K., Rogers, L. M., and Kolattukudy, P. E. 1993. Chemical signals from avocado surface wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides*. Plant Physiol. 103:267-272.
Pueppke, S. G., and VanEtten, H. D. 1974. Pisatin accumulation and lesion development in peas infected with *Aphanomyces eutreiches*, *Fusarium solani* f. sp. *pisi*, of *Rhizoctonia solani*. Phytopathology 64:1433-1440.
Recourt, K., Schripsema, J., Kijne, J. W., Brussel, A. A. N.-v., and Lugtenberg, B. J. J.1991. Inoculation of *Vicia sativa* subsp. nigra roots with *Rhizobium leguminosarum* biovar viciae results in release of nod gene activating flavanones and chalcones. Plant Mol. Biol. 16:841-852.
Rommeswinkel, M., Karwatzki, B., Beerhues, L., and Wiermann, R. 1992. Immunofluorescence localization of chalcone synthase in roots of *Pisum sativum* L. and *Phaseolus vulgaris* L. and comparable immunochemical analysis of chalcone synthase from pea leaves. Protoplasma 166:115-121.
Schäfer, W., Straney, D., Ciuffetti, L., VanEtten, H. D., and Yoder, O. C. 1989. One enzyme makes a fungal pathogen, but not a saprophyte, virulent on a new host plant. Science 246:247-249.
Schmidt, P. E., Parniske, M., and Werner, D. 1992. Production of the phytoalexin glyceollin I by soybean roots in response to symbiotic and pathogenic infection. Bot. Acta 105:18-25.
Schroth, M. N., Tousson, T. A., and Snyder, W. C. 1963. Effect of certain constituents of bean exudate on germination of chlamydospores of *Fusarium solani* f. sp. *phaseoli* in soil. Phytopathology 53:809-812.
Schuerger, A. C., Mitchell, D. J., and Kaplan, D. T. 1993. Influence of carbon source on attachment and germination of macroconidia of *Fusarium solani* f. sp. *phaseoli* on roots of *Vigna radiata* grown in hydroponic nutrient solution. Phytopathology 83:1171-1177.
Short, G. E., and Lacy, M. L. 1974. Germination of *Fusarium solani* f. sp. *pisi* chlamydospores in the spermospohere of pea. Phytopathology 64:558-562.
Short, G. E., and Lacy, M. L. 1976. Carbohydrate exudation from pea seeds: Effect of cultivar, seed age, seed color, and temperature [and relation to fungal rots]. Phytopathology 66:182-187.
Smith, D. A. 1982. Toxicity of phytoalexins: Fungitoxic activities. Pages 218-252 in: Phytoalexins. J. A. Bailey and J. W. Mansfield, eds. Blackie, Glasgow.
Stahl, D. J., and Schäfer, W. 1992. Cutinase is not required for fungal pathogenicity on pea. Plant Cell 4:621-629.
Thevelein, J. M. 1991. Fermentable sugars and intracellular acidification as specific activators of the RAS-adenylate cyclase signalling pathway in yeast: The relationship to nutrient-induced cell cycle control. Mol. Microbiol. 5:1301-1307.
Tsai, S. M., and Phillips, D. A. 1991. Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores in vitro. Appl. Environ. Microbiol. 57:1485-1488.
VanEtten, H. D., and Barz, W. 1981. Expression of pisatin demethylase ability in *Nectria haematococca*. Arch. Microbiol. 129:56-60.
VanEtten, H. D., Matthews, D. E., and Matthews, P. S. 1989. Phytoalexin detoxification: Importance for pathogenicity and practical considerations. Annu. Rev. Phytopathol. 27:143-164.
VanEtten, H. D., Pueppke, S. G., and Kelsey, T. C. 1975. 3,6a-Dihydroxy-8,9-methylene-dioxypterocarpan as a metabolite of pisatin produced by *Fusarium solani* f. sp. *pisi*. Phytochemistry 14:1103-1105.
VanEtten, H. D., and Stein, J. I. 1978. Differential response of *Fusarium solani* isolates to pisatin and phaseollin. Phytopathology 68:1552-1556.
Yarden, O., Plamann, M., Ebbole, D. J., and Yanofsky, C. 1992. *cot*-1, A gene required for hyphal elongation in *Neurospora crassa*, encodes a protein kinase. EMBO J. 11:2159-2166.
Zaat, S. A. J., Schripsema, J., Wijffelman, C. A., van Brussel, A. A. N., and Lugtenberg, B. J. J. 1989. Analysis of the major inducers of the *Rhizobium nod*A promoter from *Vicia sativa* root exudate and their activity with different *nod*D genes. Plant Mol. Biol. 13:175-188.