Isoflavonoid-Inducible Resistance to the Phytoalexin Glyceollin in Soybean Rhizobia

MARTIN PARNISKE,* BETTINA AHLBORN, AND DIETRICH WERNER

*Fachbereich Biologie der Philipps-Universität, Karl-von-Frisch-Strasse, D-3550 Marburg/Lahn,
Federal Republic of Germany*

The antibacterial effect of the soybean phytoalexin glyceollin was assayed using a liquid microculture technique. Log-phase cells of *Bradyrhizobium japonicum* and *Sinorhizobium fredii* were sensitive to glyceollin. As revealed by growth rates and survival tests, these species were able to tolerate glyceollin after adaptation. Incubation in low concentrations of the isoflavones genistein and daidzein induced resistance to potentially bactericidal concentrations of glyceollin. This inducible resistance is not due to degradation or detoxification of the phytoalexin. The inducible resistance could be detected in *B. japonicum* 110*spc*4 and 61A101, representing the two taxonomically divergent groups of this species, as well as in *S. fredii* HH103, suggesting that this trait is a feature of all soybean-nodulating rhizobia. Glyceollin resistance was also inducible in a *nodD₁D₂YABC* deletion mutant of *B. japonicum* 110*spc*4, suggesting that there exists another recognition site for flavonoids besides the *nodD* genes identified so far. Exudate preparations from roots infected with *Phytophthora megasperma* f. sp. *glycinea* exhibited a strong bactericidal effect toward glyceollin-sensitive cells of *B. japonicum*. This killing effect was not solely due to glyceollin since purified glyceollin at concentrations similar to those present in exudate preparations had a much lower toxicity. However, glyceollin-resistant cells were also more resistant to exudate preparations than glyceollin-sensitive cells. Isoflavonoid-inducible resistance must therefore be ascribed an important role for survival of rhizobia in the rhizosphere of soybean roots.

The gram-negative, slow-growing soil bacterium *Bradyrhizobium japonicum* as well as the fast-growing *Sinorhizobium fredii* (3) are able to form effective nitrogen-fixing symbioses with the host plant *Glycine max*. During the early stages of interaction between host and microsymbiont, rhizobia act very much like plant pathogens: they have to invade the root hairs of the host, thereby overcoming possible plant defense responses. Analogies between the early stages of the *Rhizobium*-legume symbiosis and a pathogenic interaction have been pointed out by several investigators (8, 9, 21, 38, 42).

In a number of plant-pathogen interactions, the induction of phytoalexins, plant-derived antimicrobial low-molecular-weight molecules, is believed to play an essential part in host resistance (10, 43). In several legumes, phytoalexins belong to the isoflavonoid type (e.g., pisatin in *Pisum* sp.; glyceollin in *Glycine* sp.; medicarpin in *Medicago* sp.). This is of special interest because flavonoids have been shown to be regulators of rhizobial *nod* gene activity (see reference 25 for a review). Considering the microsymbionts of soybean, the most active inducing compounds are isoflavonoids (1, 14, 22). Despite this intriguing bifunctionality of isoflavonoids, i.e., regulatory substances and phytoalexins, only a few data are available concerning the significance of phytoalexins in the interaction between rhizobia and their legume host. Pankhurst and co-workers studied the inhibition of rhizobia by isoflavonoids (28) as well as by flavolans (31). They found an ineffective *Rhizobium* strain to be more sensitive to the root flavolan of the host plant *Lotus corniculatus* than its effective counterpart (30). In addition, the ineffective strain induced the synthesis of higher flavolan concentrations in the nodules of this plant (29). Taken together, these results indicate the importance of flavonoids as toxic compounds for

rhizobia, which may have consequences for the symbiotic interaction.

Glyceollin, a phytoalexin of soybean (Fig. 1), is strongly induced in response to a number of biological, chemical, and physical stresses, e.g., infection with pathogenic fungi, the presence of heavy metals, or UV radiation (10, 19, 45). In symbiotic interactions, the accumulation of glyceollin has also been observed. Morandi et al. (26) found higher concentrations of glyceollin in mycorrhiza-infected roots of *G. max* than in axenic roots. Glyceollin accumulation may also take place in later stages of the *Glycine-Bradyrhizobium* interaction: nodule cells of *Glycine* sp. display a hypersensitive reaction when infected with incompatible or mutant strains of *Bradyrhizobium* sp. (32, 34, 46). The work of several laboratories confirms the assumption that, even in very early stages of the interaction, rhizobial infection contributes to enhanced release of flavonoids and phytoalexins by the host plant. Recourt et al. (37) found an increase in the number of flavonoids exuded by *Vicia sativa* in response to infection with a compatible *Rhizobium leguminosarum* strain. Enhanced levels of glyceollin could be detected in the rhizosphere of *G. max* 20 h after infection with *B. japonicum* (33, 39a).

It is very likely that soybean roots produce considerable amounts of glyceollin under field conditions due to the widespread presence of stress factors that induce defense reactions. High glyceollin levels are a challenge for microorganisms living in the rhizosphere, including symbiotic bacteria. In this study, we analyzed the effect of a phytoalexin of the host plant on the microsymbionts of soybean.

MATERIALS AND METHODS

Microbial strains. *B. japonicum* USDA 110*spc*4 (17) and 61A101 (Nitragin Co., Milwaukee, Wis.) and *S. fredii* HH103 (B. Bohlool, Paia, Hawaii) were used as wild-type strains.
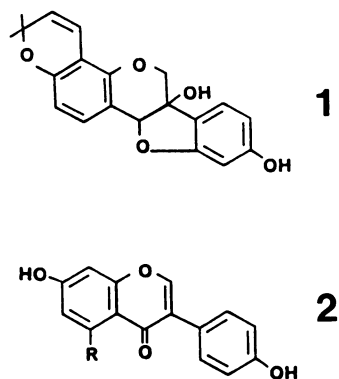
---

* Corresponding author.

FIG. 1. Structures of the soybean phytoalexin glyceollin I (1) and the isoflavones daidzein (R = H) and genistein (R = OH) (2).

The nodD$_1$D$_2$YABC deletion mutant Δ1240 and the nifD::Tn5 insertion mutant A3 (17) of B. japonicum 110spc4 were supplied by M. Göttfert and H. Hennecke, Zürich, Switzerland. Phytophthora megasperma f. sp. glycinea race 1 was obtained from T. Waldmüller and J. Ebel, Freiburg, Federal Republic of Germany.

Isolation and analysis of glyceollin. Glyceollin was isolated by the method of Grisebach (16), with the modification that glyceollin isomers I to III were collected after high-pressure liquid chromatographic (HPLC) separation from other flavonoids. The identity of glyceollin isomers was confirmed by the UV spectra and mass spectra of their trimethylsilyl derivatives.

HPLC analysis of media and culture supernatants containing glyceollin was performed under isocratic conditions using a 5-μm RP-18 column (Serva, Heidelberg, Federal Republic of Germany) with methanol-water (50:50, vol/vol) as the solvent system. The analysis of root exudates was done with a linear gradient from 100% water to 70% acetonitrile over 32 min. After HPLC separation, glyceollin concentration was determined with a UV detector and an integrator, using an ε$_{280}$ of 10,000.

Glyceollin isomers were separated by two-dimensional thin-layer chromatography, using Silica Gel 60 nano-HPTLC plates (10 by 10 cm) with fluorescent indicator (Macherey-Nagel, Düren, Federal Republic of Germany). The solvent system for the first dimension was chloroform-methanol-formic acid (93:6:1, vol/vol), and that for the second dimension was toluol-acetic acid-water (125:72:3, vol/vol). A radioimmunoassay specific for glyceollin I was performed as previously described (34).

Growth of bacteria and microculture inhibition assay. Bacteria were grown at 28°C in 20E medium (47) containing yeast extract, glycerol, mannitol, and salts. Where indicated, 20E medium buffered with 20 mM MES (morpholineethanesulfonic acid) (pH 6.5) (20E-MES) was used. Flavonoids were added from 10 mM stock solutions in N,N-dimethylformamide. Glyceollin-containing medium was prepared by adding the phytoalexin as an ethanolic solution. Final ethanol concentration was 0.9 to 1%.

To measure the inhibitory effect of glyceollin, we grew bacteria in Teflon-stoppered 2-ml glass vials (Renner GmbH, Dannstadt, Federal Republic of Germany) containing 0.8 to 1.2 ml of medium, supplemented with glyceollin at different concentrations. These microcultures were inoculated with 5 × 10^7 CFU/ml and incubated on a rotary shaker (150 rpm). Growth was monitored directly by measuring the optical

density at 600 nm (OD$_{600}$) of the suspension in the vials. Measurements were corrected for the optical density of vials containing only medium, giving the ΔOD$_{600}$ value. Aliquots (50 μl) were taken at varying intervals, bacteria were pelleted by centrifugation, and the glyceollin concentration in the supernatant was analyzed by HPLC.

Alternatively, the degree of resistance of bacterial cultures was tested by using viability tests. Aliquots of the cultures were adjusted to a ΔOD$_{600}$ of 0.1. Samples of 10 μl were mixed with 90 μl of 20E containing glyceollin. When indicated, 20E was replaced by 5 mM MES buffer (pH 6.5) (MES). The final concentration of glyceollin was 90 to 300 μM. After 3 h of incubation, appropriate dilutions were plated on 20E agar to estimate the number of surviving CFU.

In some experiments, a mixed inoculum was used containing equal numbers of cells of B. japonicum 110spc4 and A3. To achieve this, cultures of both strains were diluted to a ΔOD$_{600}$ of 0.1 and mixed in a 1:1 ratio. A 10-μl sample of the resulting suspension was mixed with 90 μl of MES containing either glyceollin or root exudate at different concentrations. After 3 h of incubation, appropriate dilutions were plated in parallel on 20E agar and on 20E agar supplemented with kanamycin and streptomycin (100 mg of each per liter). B. japonicum A3 could be differentiated from its parent strain B. japonicum 110spc4 by its ability to grow at these concentrations of antibiotics.

Growth of seedlings, zoospore infection, and exudate preparation. Culture and zoospore induction of P. megasperma f. sp. glycinea were performed as previously described (19). Soybean seeds (G. max cv. Kenwood) were surface sterilized by immersion in 30% H$_2$O$_2$ for 10 min, washed 10 times with water, soaked for 6 h, and washed again. The seeds were then placed on nitrogen-free nutrient agar (47) and grown for 2 days in a growth chamber at 25°C (16 h light/8 h dark). The seedlings were transferred to 2.5-ml test tubes with their roots submerged in 2.3 ml of a zoospore suspension of P. megasperma f. sp. glycinea race 1 (approximately 10^4 spores per plant). After 5 days of incubation in the growth chamber, the root exudates of 100 seedlings were pooled, adjusted to 10% methanol, and filtered successively through glass fiber and 0.2-μm-pore-size filters. The exudate was evaporated to dryness and taken up in 50% ethanol. A white, ethanol-water-insoluble precipitate was discarded. Aliquots were evaporated to dryness and redissolved in 5 mM MES buffer (pH 6.5) containing 1% ethanol, giving final test concentrations of glyceollin from 90 to 270 μM, as determined by HPLC.

RESULTS

Growth pattern of B. japonicum in the presence of glyceollin. We determined whether and to what extent B. japonicum is affected by glyceollin, a major phytoalexin of its host plant. Although a number of other assay systems to measure phytoalexin toxicity have been described, we used a liquid culture technique because this type of assay has been shown to be very sensitive (5). Due to the limited availability of glyceollin (preparations resulted in milligram amounts of the phytoalexin), we had to scale down the culture volume as much as possible. This was accomplished by applying the liquid microculture technique described in Materials and Methods.

Initial experiments revealed that at moderate concentrations of glyceollin (150 μM), the bacterial growth was inhibited by the phytoalexin but that this inhibition was transient. Log-phase cells of B. japonicum (ΔOD$_{600}$ = 0.5)
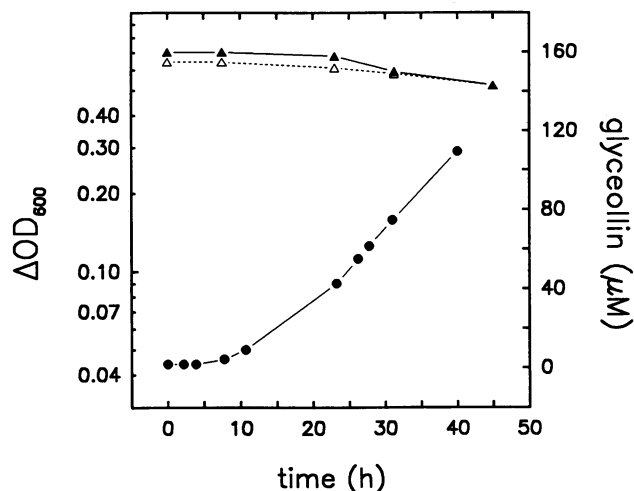
FIG. 2. Growth of *B. japonicum* 110*spc4* (●) exhibits a lag phase when it is inoculated into 150 μM glyceollin-containing medium. Glyceollin concentration, as determined by HPLC, is not affected by the growing bacteria (▲). Glyceollin concentration is also shown in a medium without added bacteria (△). Note that in Fig. 2 to 5 the ΔOD_{600} values are plotted logarithmically.

FIG. 3. Induction of glyceollin resistance by glyceollin. Growth of *B. japonicum* 110*spc4* in the presence (●) or absence (○) of 150 μM glyceollin. At the time point indicated by the arrow, aliquots of the glyceollin-grown culture were transferred to fresh medium containing either no or the same concentration of glyceollin. Note the lack of the lag phase after transfer.

were inhibited in growth when transferred to glyceollin-containing medium. At 150 μM glyceollin, a lag period of about 10 h occurred, after which the cells started to grow logarithmically (Fig. 2). This transient inhibition might be interpreted in two different ways: (i) glyceollin concentration drops to a level that is no longer inhibitory, either because of detoxification of glyceollin by *B. japonicum* or because of an instability of the compound; or (ii) the bacteria do not metabolize glyceollin but grow due to a tolerance to the phytoalexin. We performed three experiments to assess the probability of these two alternatives.

Failure of *B. japonicum* to metabolize glyceollin. In the first experiment, we tested whether *B. japonicum* is able to metabolize or detoxify glyceollin. Bacteria were grown by the microculture technique, and samples were taken at various intervals to determine glyceollin concentration in the supernatant by HPLC. Glyceollin content in the presence of growing bacteria was reduced only slightly (maximum, 13% within 48 h). This reduction was comparable with the loss of glyceollin in a control medium without bacteria (Fig. 2). Thus, we concluded that this loss is not due to bacterial metabolism.

The stability of glyceollin in the presence of rhizobia was verified by two additional analytical techniques. Glyceollin I concentration, determined by a radioimmunoassay specific for this phytoalexin, showed the same slight decrease in the presence or absence of bacteria. Two-dimensional thin-layer chromatography of ethyl acetate extracts isolated from a medium in which bacteria had already reached the stationary phase did not show differences in either the total amount or the ratio of the glyceollin isomers compared to the originally introduced substance.

In a second experiment, we checked whether glyceollin keeps its biological activity in the presence of bacteria, to rule out possible chemical modifications which were not detectable by our analytical techniques. *B. japonicum* was grown to the late stationary phase in a medium with 150 μM glyceollin. The bacteria were pelleted by centrifugation, and glyceollin in the supernatant was extracted twice with ethyl
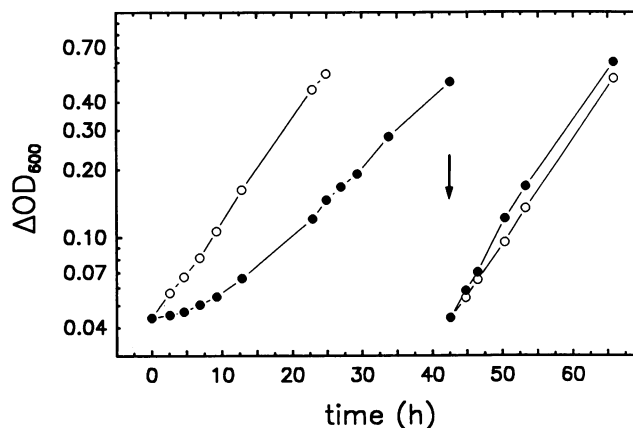
acetate. By means of the inhibition assay, it could be shown that the glyceollin recovered from the supernatant retained an inhibitory potency comparable to that of the originally introduced substance.

Tolerance of glyceollin by *B. japonicum* after adaptation. Since *B. japonicum* does not detoxify glyceollin and is nevertheless able to grow after a lag period, it appears that an adaptation has taken place that makes the cells resistant to the phytoalexin. In a third experiment, it was shown that *B. japonicum* gains glyceollin resistance during the lag phase. When cells were grown in the presence of 150 μM glyceollin, the characteristic lag phase occurred (Fig. 3). After the culture had reached the log phase, an aliquot was inoculated into fresh medium containing glyceollin at the same concentration. Growth of this culture exhibited no lag phase, and its doubling time was similar to that of the control, which was inoculated in a glyceollin-free medium (Fig. 3).

The results of the experiments described above confirmed our hypothesis that the observed growth pattern in the presence of glyceollin is not due to metabolism or detoxification of glyceollin by the bacteria but rather because *B. japonicum* becomes glyceollin resistant during incubation in a glyceollin-containing medium.

In the experiments described above, *B. japonicum* 61A101 was indistinguishable from strain 110*spc4*, indicating that resistance to glyceollin can be induced in both strains.

Induction of glyceollin resistance by flavonoids. Since resistance to glyceollin is inducible by incubation in glyceollin-containing medium, we were interested in determining whether structurally related compounds were also able to induce resistance to glyceollin. Genistein (Fig. 1), an isoflavonoid present in soybean roots (15, 33, 35), was analyzed for its capacity to induce resistance in *B. japonicum*. Figure 4 shows the effect of a genistein preculture on the growth pattern in the presence of glyceollin. Nontreated cells were strongly inhibited by the high glyceollin concentrations (300 and 440 μM) applied in this experiment. In contrast, the genistein-pretreated cells were able to grow when transferred to the glyceollin medium. This result demonstrated that glyceollin resistance of *B. japonicum* is inducible by the isoflavonoid genistein. Indeed, for a successful induction of
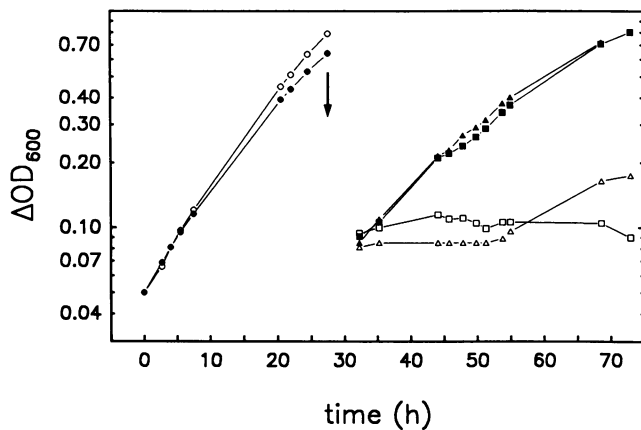
FIG. 4. Induction of glyceollin resistance by genistein. *B. japonicum* 110spc4 was precultured either in control medium or in medium containing 10 μM genistein. At the time point indicated by the arrow, aliquots were transferred to glyceollin-containing medium. ●, preculture in 10 μM genistein with transfer to 300 μM (▲) or 440 μM (■) glyceollin. ○, preculture in medium only with transfer to 300 μM (△) or 440 μM (□) glyceollin.

the resistance, much lower concentrations are needed than those necessary for the inhibitory effect. Low concentrations of genistein (10 μM) induce resistance to an at least 44-fold-higher concentration of glyceollin.

The finding that genistein induces resistance was further confirmed by viability tests. A strong bactericidal effect was observed when cells grown in control medium were exposed to 300 μM glyceollin. The number of surviving CFU declined to a minimum 3 h after transfer to a glyceollin-containing medium (data not shown). In contrast, the cells precultured in the presence of genistein were much less affected by glyceollin. The development of glyceollin resistance during growth in 10 μM genistein was analyzed in more detail. For this purpose, aliquots of growing cultures taken at certain
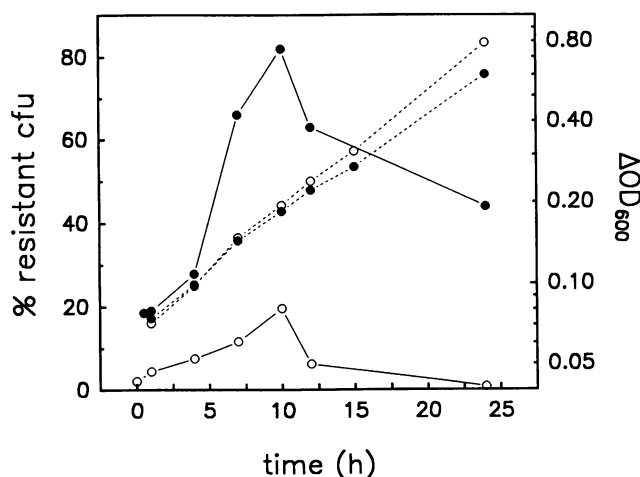

FIG. 5. Development of glyceollin resistance during growth of *B. japonicum* 110spc4 in the presence of genistein. Bacteria were grown either in control medium (○) or in medium containing 10 μM genistein (●). At various intervals, aliquots of the cultures were removed and cells were tested for survival in 20E medium containing 300 μM glyceollin. ----, growth of *B. japonicum* 110spc4. ——, degree of resistance to glyceollin.

intervals were exposed to 300 μM glyceollin and the number of surviving CFU was taken as a parameter for the degree of resistance to the phytoalexin. Figure 5 shows the kinetics of the induction of glyceollin resistance in both a control culture and a culture grown in the presence of genistein. The culture with genistein very quickly acquired a significant degree of resistance. Already after 30 min about 20% of the cells survived the glyceollin treatment. A maximum was reached after 10 h, followed by a gradual decline in the degree of resistance. The control culture was much more sensitive to the phytoalexin, yet a weak maximum of resistance was exhibited synchronous to that of the genistein culture.

An inducible resistance such as we observe might be due either to a physiological adaptation of the cells or to the preferential growth of a preexisting genetically resistant subpopulation. The time needed for the acquisition and loss of resistance is shorter than can be explained by the shortest generation time (6 to 7 h) observed for *B. japonicum*. This indicates that glyceollin resistance is due to an inducible trait rather than to the selection of a preexisting resistant subpopulation.

Data obtained from CFU counts have to be interpreted with care since the ratio between CFU counts and living cells is not constant under all conditions. In some media, cells of *B. japonicum* tend to form large aggregates giving rise to low CFU/living cell ratios. In the medium used in this study, this tendency was low, a fact that might be attributed to the very low amounts of capsular polysaccharides that were produced. To eliminate the possibility that the observed differences in CFU counts could be attributed to differences in clump formation, we performed microscopic observations and cell counts in the inhibition assay after 3 h of incubation. These analyses revealed that about 10 to 15% of total cells counted were present in the form of aggregates of three to five cells. There were no detectable differences in clump formation between all cultures tested. Most important, the cultures with the lowest CFU recovery rates showed no altered clumping frequency. In addition, treatment of inhibition assay mixtures in an ultrasonic water bath before dilution and plating did not significantly increase the number of CFU in any of the assays.

To further test for possible aggregate formation, we did a mixed inoculum experiment in which a genetically marked strain was mixed with its parent in a 1:1 ratio and this mixture was exposed to glyceollin. Prior to the inhibition assay, the strains were grown in either the presence or absence of 20 μM genistein to obtain glyceollin-resistant or -sensitive cells, respectively. As shown in Fig. 6, sensitive cells were recovered at the same rate from the inhibition assay irrespective of whether the resistant counterpart was present or not. Likewise, the resistant strain was recovered at similar rates independent of the presence of the sensitive strain. In the range of 1×10^5 to 2×10^7 CFU/ml, the recovery rates for glyceollin-resistant and -sensitive strains in this type of assay were independent of the initial cell densities applied (data not shown). It therefore appears very unlikely that the observed differences in CFU recovery rates between the different strains are due to differential clumping effects. Clumping to an extent that it reduces the CFU recovery of the one strain more than 10-fold without affecting the recovery rate of the other strain is hard to imagine. On the basis of these results, we conclude that lower recovery rates obtained for sensitive cells are not due to aggregate formation.

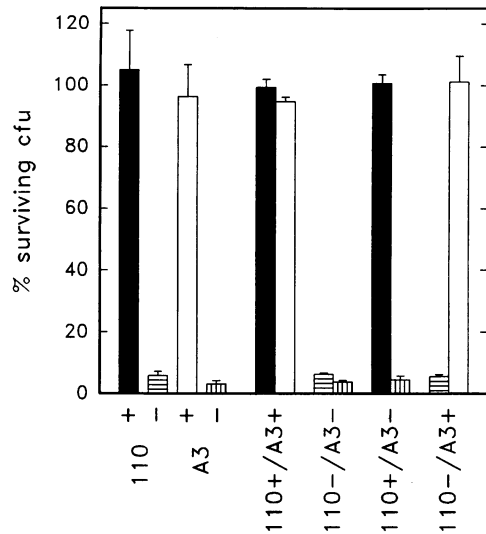These results also show that sensitive cells are not pro-

FIG. 6. Rates of recovery of CFU after exposure of single or mixed strains of *B. japonicum* to glyceollin. *B. japonicum* 110*spc*4 and the *nifD*::Tn*5* mutant A3 were precultured either in 20E-MES (−) or in medium supplemented with 20 μM genistein (+) and subsequently inoculated either as single strains (four left bars) or mixed in a 1:1 ratio (right bars) into 270 μM glyceollin in MES buffer. After 3 h of incubation, the number of CFU counts of each strain per milliliter was determined. The CFU recovery rate is given as the percentage of the CFU counts inoculated (10^7 CFU of each strain per ml). Recovery rates of strains from MES buffer without glyceollin were about 140%.

tected from the bactericidal effect of glyceollin by the presence of the resistant strain. This finding provides additional evidence that glyceollin resistance is not due to degradation or detoxification.

A number of other flavonoids were tested for their capacity to induce glyceollin resistance (Table 1). The isoflavonoids genistein and daidzein are potent inducers, while luteolin and coumestrol lack this property. Naringenin and apigenin gave very variable results (data not shown). This might be attributed to the ability of *B. japonicum* to metabolize these substances. Evidence for naringenin breakdown by *B. japonicum* 110 has been previously reported (23).

Absence of involvement of the common *nod* genes in glyceollin resistance. Since we have shown that flavonoids induce glyceollin resistance and it is known that they also interact

TABLE 1. Induction of glyceollin resistance by flavonoids

Strain	Preculture[a]	Resistance (%)[b]
B. japonicum 110*spc*4	Medium only	1.4
	0.1% DMF	1.4
	Genistein	100.0
	Daidzein	58.5
	Coumestrol	2.0
	Luteolin	1.4
S. fredii HH103	0.1% DMF	2.9
	Genistein	100.0

[a] Bacteria were precultured overnight in 20E medium containing either 10 μM of each flavonoid or dimethylformamide (DMF) only.

[b] 3 h after transfer to 20E containing bactericidal concentrations (300 μM) of glyceollin, the number of surviving CFU was determined. Resistance is expressed as the percentage of the value obtained with genistein-treated cells.
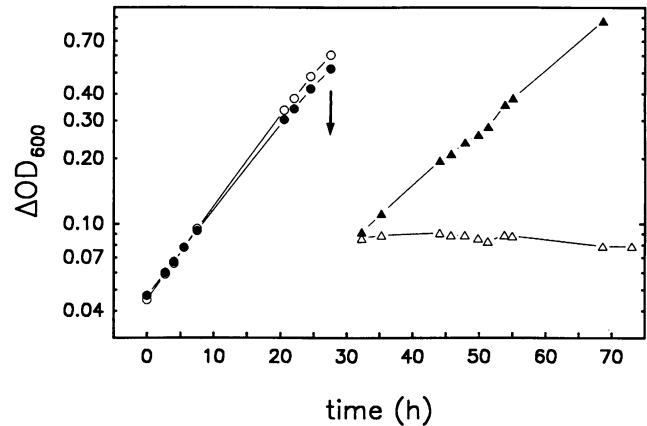

FIG. 7. Effect of a *nodD₂D₁YABC* deletion on the inducibility of glyceollin resistance by genistein. Experimental design and symbols are as described in the legend to Fig. 3, except that the *B. japonicum* mutant Δ1240 was tested and only one concentration of glyceollin was used.

with the *nodD* gene product (25), we therefore analyzed the effect of a *nodD* deletion on the inducibility of glyceollin resistance by genistein. In the *nodD₁D₂YABC* deletion mutant of strain 110*spc*4 (Δ1240), glyceollin resistance was still inducible by genistein (Fig. 7). The growth pattern of the induced cells in the presence of glyceollin was the same as that of the wild type. This result indicates that the two *nodD* genes are not necessary for induction of the resistance.

Induction of glyceollin resistance in *S. fredii* HH103. If the inducibility of glyceollin resistance is an advantage for a successful symbiosis with *G. max*, it is likely that this trait is also present in other rhizobia which are able to nodulate soybeans. Therefore, we included *S. fredii*, a species very distinct from *Bradyrhizobium* sp., but still able to nodulate *G. max* (3), in our studies. The numbers of living cells of *S. fredii* HH103 were strongly reduced 3 h after transfer from normal medium to a glyceollin-containing medium (Table 1). In contrast, genistein-precultured cells were not affected in viability by the same concentration of glyceollin. This led to the conclusion that glyceollin resistance can also be induced in *S. fredii*.

Protection of glyceollin-resistant cells against toxic compounds present in exudate from phytoalexin-producing soybean roots. It is likely that high amounts of phytoalexins are deposited in the rhizosphere of soybean roots when the plant is challenged by pathogenic organisms. Under these circumstances, resistance to phytoalexins, as observed in the present study, might be of ecological significance. To test this hypothesis, we collected exudate from soybean roots that were previously infected with zoospores of *P. megasperma* f. sp. *glycinea*. HPLC analysis revealed that glyceollin was a major component of this exudate. Five days after infection, 10 to 20 nmol of glyceollin per root was obtained from the compatible interaction of cultivar Kenwood with *P. megasperma* f. sp. *glycinea* race 1. It is known that roots of soybean react with a massive accumulation of phytoalexin upon infection with zoospores of *P. megasperma* f. sp. *glycinea* (19). Here we observed that glyceollin also accumulates in the rhizosphere of such seedlings. When *B. japonicum* cells were exposed to root exudate, it was observed (Fig. 8) (i) that as many as 3 orders of magnitude more glyceollin-resistant cells as sensitive cells
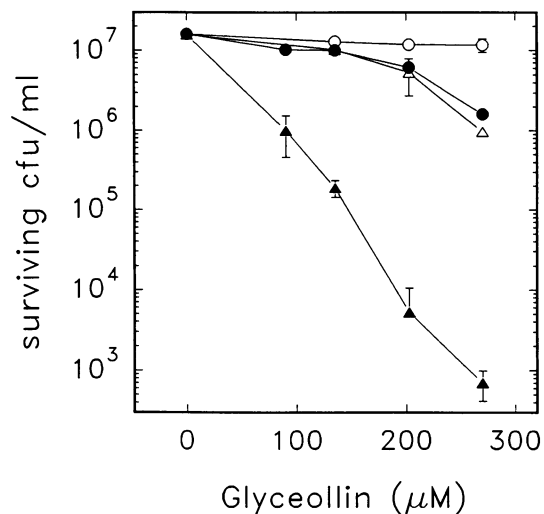
FIG. 8. Inhibition of *B. japonicum* cells by glyceollin or root exudate. Root exudate was concentrated to glyceollin concentrations between 90 and 270 μM (●, ▲). Its inhibitory effect was compared with that of purified glyceollin in MES buffer (O, △). A mixed inoculum (10⁷ CFU of each per ml) of strain 110*spc*4 (●, O) and strain A3 (▲, △) was used. Prior to the inhibition assay, strain 110*spc*4 was precultured overnight in 20E-MES supplemented with 20 μM genistein to induce glyceollin resistance, while strain A3 was grown in 20E-MES only. After 3 h of incubation, the number of surviving CFU of each strain was determined.

were recovered and (ii) that exudate concentrated to a defined glyceollin content exhibited a severalfold-higher toxicity than a similar concentration of purified glyceollin. Therefore, the toxic effect of the exudate cannot solely be ascribed to the presence of glyceollin. Consequently, there must be additional factors in this exudate that are either toxic or increase the toxicity of glyceollin. HPLC analysis demonstrated that soybeans exude a mixture of isoflavonoids and other substances that might interfere with the toxicity of glyceollin. These results obtained with root exudate indicate a possible ecological significance of the inducible glyceollin resistance. It is likely that this trait enhances the ability of the microsymbionts to survive in the rhizosphere of phytoalexin-producing host roots.

DISCUSSION

The results of this study show that rhizobia which form symbioses with soybeans are susceptible to the phytoalexin glyceollin produced by their host plant. More interestingly, all soybean rhizobia tested are able to adapt to the presence of the phytoalexin and tolerate previously bactericidal concentrations. Low concentrations of isoflavones can induce glyceollin resistance. These symbiotic signal compounds are constitutively present in the host plant rhizosphere (4, 15, 22, 33, 35).

Furthermore, the exudate from phytoalexin-producing soybean roots contains a toxic mixture of compounds that are able to kill cells of the microsymbiont. The killing effect of this exudate is not only due to the presence of glyceollin. However, glyceollin-resistant cells are much more resistant to this mixture than sensitive ones. We conclude that one important function of the inducible resistance is to increase the survival of the microsymbiont in the rhizosphere of challenged host roots.

Phytoalexin accumulation upon contact with incompatible pathogenic microorganisms is a local response. Only plant cells at or in the vicinity of the infection site react with a massive production of phytoalexins (19). Field-grown soybean roots should have multiple sites of phytoalexin accumulation due to the presence of pathogenic organisms and other environmental stress factors that elicit defense responses. It is therefore plausible that the rhizosphere of soybeans consists of temporal as well as spatial concentration gradients of phytoalexins. An inducible resistance would allow cells of the microsymbiont the physiological plasticity to adapt to changes in phytoalexin concentration.

It is not yet clear whether phytoalexin resistance confers any advantages other than the ability to survive in the rhizosphere of phytoalexin-producing plants. Reports of enhanced flavonoid or phytoalexin concentrations at several different steps of the symbiotic legume-*Rhizobium* interaction after infection with compatible (29, 33, 37), incompatible (29, 34, 46), or mutant (9, 32) rhizobia suggest that phytoalexin resistance could also be important at later stages of the interaction.

B. japonicum 110 and 61A101 represent two highly divergent groups within this bacterial species (7, 18, 40). The fast-growing microsymbiont of soybeans, *S. fredii*, is very little related to the bradyrhizobia (3). Yet, all soybean rhizobia tested exhibited the phenotype of inducible resistance. The apparent conservation of this phenotype between these relatively unrelated rhizobia suggests an essential role in the symbiotic interaction with the host plant.

In the rhizosphere of *G. max*, isoflavones are constitutively present (4, 15, 22, 33, 35). These isoflavones have at least two functions in the interaction between *G. max* and its microsymbionts: (i) they induce the common *nod* genes (1, 14, 20, 22) as well as *B. japonicum* genes for host-specific nodulation of soybeans (7); and (ii) they apparently also induce the resistance against the phytoalexin glyceollin. The *nodD* gene product is believed to interact with flavonoids, thereby activating the transcription of the common *nod* genes (25). However, in the common *nod* mutant Δ1240, tolerance to glyceollin is still inducible, indicating that these genes are not required for the expression of the resistance. Thus, a recognition process specifically activated by isoflavonoids not involving the previously described *nodD* genes has to be postulated in *B. japonicum*.

In fungal species, inducible phytoalexin resistance without degradation of the phytoalexin has already been observed. Denny and VanEtten (5, 6) reported an inducible resistance to pisatin in the ascomycete *Nectria haematococca*. Stössel (41) observed an adaptation of the oomycete *P. megasperma* to glyceollin. The phenomena described by these researchers share similarities with those presented here. In all these cases, resistance to phytoalexins of the isoflavonoid type is inducible by isoflavonoids. In addition, an inducible resistance of fungi to a phytoalexin of carrots has been observed (24). Our data constitute the first report of an inducible phytoalexin resistance in a bacterial species.

We were unable to detect degradation of glyceollin by *B. japonicum*, *Rhizobium meliloti*, *R. leguminosarum*, or *Erwinia carotovora* (data not shown). Fett and Osman (11) found that glyceollin was not degraded by *Pseudomonas syringae* subsp. glycinea. Stössel (41) observed no metabolism of glyceollin by *P. megasperma*. To our knowledge, there are no reports of degradation of glyceollin by microorganisms. It is known that metabolism of phytoalexins might be influenced by the media and solvents used (see, for example, reference 5). Therefore, it cannot be excluded that

the lack of metabolism must be attributed to the special conditions applied in these studies. However, all these findings taken together suggest a particular stability of this molecule against degradation. Interestingly, the less complex pterocarpan glycinol, a biosynthetic precursor of glyceollin, has been reported to be metabolized by *E. carotovora* (45). Phytoalexin detoxification has been proposed as an important mechanism by which pathogens overcome this plant defense response (43). The apparent difference in degradability of glycinol and glyceollin might be one explanation for the evolution of more complex phytoalexins which are not so easily detoxified by pathogenic microorganisms.

The mode by which glyceollin and other pterocarpan phytoalexins exert their antibiotic activity has been the subject of a number of studies. While some results suggest a nonspecific mode of action involving disruption of membrane integrity (44), other data support the hypothesis that glyceollin works via specific sensitive target enzymes such as ATPase (12) or NADH-ubiquinone-oxidoreductase (2). From unpublished data, we know that the NADH-ubiquinone-oxidoreductase of *B. japonicum* 110*spc*4 is highly sensitive to glyceollin, being strongly affected at concentrations as low as 2 μM. The nonspecific membrane effects of glyceollin were observed at much higher concentrations (44). Therefore, it appears that the inhibition of specific target enzymes might be the primary mode of the antibiotic effect of glyceollin. However, as shown in the present study, glyceollin-resistant *B. japonicum* cells tolerate concentrations of glyceollin up to 440 μM. Such high concentrations have been reported to disturb membrane integrity (44).

With our present knowledge, we can only speculate about the mechanism by which soybean rhizobia overcome the inhibitory effect of glyceollin. The nonspecific mode of action via membrane disintegration makes an exclusion mechanism more likely to be responsible for the resistance than an adaptation of a target enzyme. It is well established that the outer membrane of gram-negative bacteria acts as a permeability barrier for hydrophobic substances (27). Gram-positive bacteria exhibit a much higher sensitivity to isoflavonoids than gram-negative ones (13). On the basis of these observations, we propose that the outer membrane with its limited permeability for hydrophobic compounds is involved in the resistance against glyceollin. However, if the resistance confers a significant advantage on the microsymbiont, the question remains open as to why a sensitive status exists at all. Flavonoid *nod* gene inducers are thought to accumulate in the cytoplasmic membrane (36) where the *nodD* gene product is located (39). To achieve an induction, obviously these substances have to pass the outer membrane. Herein lies the dilemma for rhizobia. On the one hand, flavonoid compounds, even when present at very low concentrations, have to be taken up as signal compounds. On the other hand, an inhibitory intracellular concentration of isoflavonoids must not be reached. Permeability changes of the outer membrane, induced by isoflavonoids, would be a plausible, yet to be tested, solution for these divergent requirements as well as an explanation for the effects observed.

ACKNOWLEDGMENTS

REFERENCES

1. **Banfalvi, Z., A. Nieuwkoop, M. Schell, L. Besl, and G. Stacey.** 1988. Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. **214**:420–424.
2. **Boydston, R., J. D. Paxton, and D. E. Koeppe.** 1983. Glyceollin: a site-specific inhibitor of electron transport in isolated soybean mitochondria. Plant Physiol. **72**:151–155.
3. **Chen, W. X., G. H. Yan, and J. L. Li.** 1988. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int. J. Syst. Bacteriol. **38**:392–397.
4. **D'Arcy-Lameta, A.** 1986. Study of soybean and lentil root exudates. II. Identification of some polyphenolic compounds, relation with plantlet physiology. Plant Soil **92**:113–123.
5. **Denny, T. P., and H. D. VanEtten.** 1983. Tolerance of *Nectria haematococca* MP VI to the phytoalexin pisatin in the absence of detoxification. J. Gen. Microbiol. **129**:2893–2901.
6. **Denny, T. P., and H. D. VanEtten.** 1983. Characterization of an inducible, nondegradative tolerance of *Nectria haematococca* MP VI to phytoalexins. J. Gen. Microbiol. **129**:2903–2913.
7. **Deshmane, N., and G. Stacey.** 1989. Identification of *Bradyrhizobium japonicum nod* genes involved in host-specific nodulation. J. Bacteriol. **171**:3324–3330.
8. **Djordjevic, M. A., D. W. Gabriel, and B. G. Rolfe.** 1987. *Rhizobium*—the refined parasite of legumes. Annu. Rev. Phytopathol. **25**:145–168.
9. **Djordjevic, S. P., R. W. Ridge, H. Chen, J. W. Redmond, M. Batley, and B. G. Rolfe.** 1988. Induction of pathogenic-like responses in the legume *Macroptilium atropurpureum* by a transposon-induced mutant of the fast-growing, broad-host-range *Rhizobium* strain NGR234. J. Bacteriol. **170**:1848–1857.
10. **Ebel, J., and H. Grisebach.** 1988. Defense strategies of soybean against the fungus *Phytophthora megasperma* f. sp. *glycinea*: a molecular analysis. Trends Biochem. Sci. **13**:23–27.
11. **Fett, W. F., and S. F. Osman.** 1982. Inhibition of bacteria by the soybean isoflavonoids glyceollin and coumestrol. Phytopathology **72**:755–760.
12. **Giannini, J. I., D. P. Briskin, J. S. Holt, and J. D. Paxton.** 1988. Inhibition of plasma membrane and tonoplast H$^+$-transporting ATPases by glyceollin. Phytopathology **78**:1000–1003.
13. **Gnanamanickam, S. S., and D. A. Smith.** 1980. Selective toxicity of isoflavonoid phytoalexins to gram-positive bacteria. Phytopathology **70**:894–896.
14. **Göttfert, M., J. Weber, and H. Hennecke.** 1988. Induction of a *nodA-lacZ* fusion in *Bradyrhizobium japonicum* by an isoflavone. J. Plant Physiol. **132**:394–397.
15. **Graham, T. L.** 1991. Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. Plant Physiol. **95**:594–603.
16. **Grisebach, H.** 1986. Isolation of glyceollin I, p. 75–76. *In* H. F. Linskens and J. F. Jackson (ed.), Modern methods of plant analysis, new series, vol. 4: Immunology in plant sciences. Springer-Verlag KG, Berlin.
17. **Hahn, M., and H. Hennecke.** 1984. Localized mutagenesis in *Rhizobium japonicum*. Mol. Gen. Genet. **193**:46–52.
18. **Hahn, M., and H. Hennecke.** 1987. Conservation of a symbiotic DNA region in soybean root nodule bacteria. Appl. Environ. Microbiol. **53**:2253–2255.
19. **Hahn, M. G., A. Bonhoff, and H. Grisebach.** 1985. Quantitative localization of the phytoalexin glyceollin I in relation to fungal hyphae in soybean roots infected with *Phytophthora megasperma* f. sp. *glycinea*. Plant Physiol. **77**:591–601.
20. **Kape, R., M. Parniske, and D. Werner.** 1991. Chemotaxis and *nod* gene activity of *Bradyrhizobium japonicum* in response to hydroxycinnamic acids and isoflavonoids. Appl. Environ. Microbiol. **57**:316–319.
21. **Keen, N. T., and B. Staskawicz.** 1988. Host range determinants in plant pathogens and symbionts. Annu. Rev. Microbiol. **42**:421–440.
22. **Kosslak, R. M., R. Bookland, J. Barkei, H. E. Paaren, and E. R. Appelbaum.** 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. Proc. Natl. Acad. Sci. USA **84**:7428–7432.

23. Kosslak, R. M., R. S. Joshi, B. A. Bowen, H. E. Paaren, and E. R. Appelbaum. 1990. Strain-specific inhibition of *nod* gene induction in *Bradyrhizobium japonicum* by flavonoid compounds. Appl. Environ. Microbiol. 56:1333–1341.

24. Kurosaki, F., I. Sakurai, and A. Nishi. 1984. Development of tolerance to the phytoalexin 6-methoxymellein in microorganisms. J. Gen. Appl. Microbiol. 30:43–52.

25. Long, S. R. 1989. *Rhizobium*-legume nodulation: life together in the underground. Cell 56:203–214.

26. Morandi, D., J. A. Bailey, and V. Gianinazzi-Pearson. 1984. Isoflavonoid accumulation in soybean roots infected with vesicular-arbuscular mycorrhizal fungi. Physiol. Plant Pathol. 24:357–364.

27. Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49:1–32.

28. Pankhurst, C. E., and D. R. Biggs. 1980. Sensitivity of *Rhizobium* to selected isoflavonoids. Can. J. Microbiol. 26:542–545.

29. Pankhurst, C. E., A. S. Craig, and W. T. Jones. 1979. Effectiveness of *Lotus* root nodules. I. Morphology and flavolan content of nodules formed on *Lotus pedunculatus* by fast-growing *Lotus* rhizobia. J. Exp. Bot. 30:1085–1093.

30. Pankhurst, C. E., and W. T. Jones. 1979. Effectiveness of *Lotus* root nodules. II. Relationship between root nodule effectiveness and '*in vitro*' sensitivity of fast-growing *Lotus* rhizobia to flavolans. J. Exp. Bot. 30:1095–1107.

31. Pankhurst, C. E., W. T. Jones, and A. S. Craig. 1982. Bactericidal effect of *Lotus pedunculatus* root flavolan on fast-growing *Lotus* rhizobia. J. Gen. Microbiol. 128:1567–1576.

32. Parniske, M., H. M. Fischer, H. Hennecke, and D. Werner. 1991. Z. Naturforsch, in press.

33. Parniske, M., G. Pausch, and D. Werner. 1988. Changes in flavonoid pattern of root hairs of *Glycine max* in response to symbiotic infection with *Bradyrhizobium japonicum*, p. 466. *In* H. Bothe, F. J. de Bruijn, and W. E. Newton (ed.), Nitrogen fixation: hundred years after. G. Fischer Verlag, Stuttgart.

34. Parniske, M., C. Zimmermann, P. B. Cregan, and D. Werner. 1990. Hypersensitive reaction of nodule cells in the *Glycine sp./Bradyrhizobium japonicum*-symbiosis occurs at the genotype-specific level. Bot. Acta 103:143–148.

35. Porter, P. M., W. L. Banwart, and J. J. Hassett. 1985. HPLC isolation and GC-MS identification of genistein, daidzein, and coumestrol from unhydrolysed soybean root extracts. Environ. Exp. Bot. 25:229–232.

36. Recourt, K., A. A. N. van Brussel, A. J. M. Driessen, and B. J. J. Lugtenberg. 1989. Accumulation of a *nod* gene inducer, the flavonoid naringenin, in the cytoplasmic membrane of *Rhizobium leguminosarum* biovar *viciae* is caused by the pH-dependent hydrophobicity of naringenin. J. Bacteriol. 171:4370–4377.

37. Recourt, K., A. A. N. van Brussel, J. Kijne, J. Schripsema, and B. J. J. Lugtenberg. 1990. Inoculation of *Vicia sativa* ssp. *nigra* with *Rhizobium leguminosarum* bv. *viciae* increases the number of *nod* gene inducing flavonoids released by the roots of the host plant, p. 271. *In* P. M. Gresshoff, L. E. Roth, G. Stacey, and W. E. Newton (ed.), Nitrogen fixation: achievements and objectives. Chapman & Hall, New York.

38. Rolfe, B. G., and P. M. Gresshoff. 1988. Genetic analysis of legume nodule initiation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39:297–319.

39. Schlaman, H. R. M., H. P. Spaink, R. J. H. Okker, and B. J. J. Lugtenberg. 1989. Subcellular localization of the *nodD* gene product in *Rhizobium leguminosarum*. J. Bacteriol. 171:4686–4693.

39a.Schmidt, P. E., M. Parniske, and D. Werner. Unpublished data.

40. Stanley, J., G. S. Brown, and D. P. S. Verma. 1985. Slow-growing *Rhizobium japonicum* comprises two highly divergent symbiotic types. J. Bacteriol. 163:148–154.

41. Stössel, P. 1983. In vitro effects of glyceollin on *Phytophthora megasperma* f. sp. *glycinea*. Phytopathology 73:1603–1607.

42. Vance, C. P. 1983. *Rhizobium* infection and nodulation: a beneficial plant disease? Annu. Rev. Microbiol. 37:399–424.

43. Van Etten, H. D., D. E. Matthews, and P. S. Matthews. 1989. Phytoalexin detoxification: importance for pathogenicity and practical implications. Annu. Rev. Phytopathol. 27:143–164.

44. Weinstein, L. I., and P. Albersheim. 1983. Host-pathogen interactions. XXIII. The mechanism of the antibacterial action of glycinol, a pterocarpan phytoalexin synthesized by soybeans. Plant Physiol. 72:557–563.

45. Weinstein, L. I., M. G. Hahn, and P. Albersheim. 1981. Host-pathogen interactions. XVIII. Isolation and biological activity of glycinol, a pterocarpan phytoalexin synthesized by soybeans. Plant Physiol. 68:358–363.

46. Werner, D., R. B. Mellor, M. G. Hahn, and H. Grisebach. 1985. Soybean root response to symbiotic infection. Glyceollin I accumulation in an ineffective type of soybean nodules with an early loss of the peribacteroid membrane. Z. Naturforsch. 40c:179–181.

47. Werner, D., J. Wilcockson, and E. Zimmermann. 1975. Adsorption and selection of rhizobia with ion-exchange papers. Arch. Microbiol. 105:27–32.