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Use the following prefixes to names of units

giga (10^9)	G	micro (10^{-6})	μ
mega (10^6)	M	nano (10^{-9})	n
kilo (10^3)	k	pico (10^{-12})	p
milli (10^{-3})	m	femto (10^{-15})	f

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103 Tables

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Subject Index

Production of the Phytoalexin Glyceollin I by Soybean Roots in Response to Symbiotic and Pathogenic Infection

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Abstract

The amount of the phytoalexin glyceollin I in root exudate and root hairs of individual seedlings of *Glycine max* (L. Merr. cv. Preston) was analysed using a radioimmunoassay. *Bradyrhizobium japonicum* 110*spc*4, which is able to form nitrogen fixing nodules with this plant, caused an increase of up to 50-fold in glyceollin I levels in root exudate relative to uninfected control seedlings. Maximum glyceollin I levels were reached within 10 h of incubation. Elevated glyceollin I levels were also observed after incubation of soybean roots in sterile bacterial supernatant, a suspension of autoclaved bacteria or the supernatant from broken cells of *Bradyrhizobium japonicum*. Increased glyceollin I production is not due to the process of active root hair penetration by the microsymbiont since living bacterial cells are not necessary for the induction. The observed glyceollin I production in response to *Bradyrhizobium japonicum* is several times lower than that after pathogenic infection. Infection with zoospores of the phytopathogenic oomycete, *Phytophthora megasperma* f. sp. *glycinea* race 1, leads within 20 h to an accumulation of 7 nmol glyceollin I/seedling in the root exudate of the compatible cultivar Kenwood and 48 nmol glyceollin I/seedlings in that of the incompatible cultivar Maple Arrow. These results support the idea that phytoalexins are implicated in determination of compatibility in pathogenic interactions. Crude cell extracts of different symbiotic bacteria (*Bradyrhizobium japonicum* 110*spc*4, *Rhizobium meliloti* 2011, *Rhizobium leguminosarum* PRE 8, *Sinorhizobium fredii* HH 103) were found to induce different amounts of glyceollin I in the root exudate. The observed glyceollin I levels could not be correlated with the ability of these rhizobial strains to nodulate *Glycine max*. Inhibition of flavonoid and phytoalexin synthesis by (R)-(1-amino-2-phenylethyl)phosphonic acid (APEP), a specific inhibitor of the phenylalanine-ammonia-lyase (PAL), during the first 20 h of the symbiotic interaction dramatically decreased the number of nodules formed in root regions that had been in contact with the inhibitor. This effect was observed at concentrations that inhibited neither bacterial nor plant growth. The implications of these findings for the process of nodule initation are discussed.

Key words

Flavonoids, nodule initiation, *Glycine max*, *Bradyrhizobium japonicum*, *Phytophthora megasperma* f. sp. *glycinea*, root exudate, root hairs.

Abbreviations and Symbols

APEP:	(R)-(1-amino-2-phenylethyl)phosphonic acid
cfu:	colony forming units
CHS:	chalcone synthase
MES:	5 mM morpholinethanesulfonic acid, 1 mM $CaCl_2$, pH 6.2
PAL:	phenylalanine-ammonia-lyase
rtm:	root tip mark
XMM:	xylose minimal medium

Introduction

The symbiosis between the soybean *Glycine max* and the Gram-negative soil bacterium *Bradyrhizobium japonicum* (110*spc*4) results in the formation of root nodules in which the bacteria actively fix atmospheric nitrogen. It is well established that the early stages of the rhizobia-legume interaction can be subdivided into two fundamentally different physiological processes (Long, 1989): on the one hand, rhizobia infect root hairs and thereby resemble plant pathogens invading host tissues (Djordjevic et al., 1987). On the other hand, rhizobia induce changes in the developmental program of root cells so that they become meristematic (Rolfe and Gresshoff, 1988).

One group of secondary plant products, the flavonoids, has been implicated both in plant defense reactions as well as in plant developmental processes. The isoflavonoid glyceollin I is an antibiotic phytoalexin found in *Glycine max*. Phytoalexins are considered to play an important role in pathogenic interactions as an early plant defense response (Hahn et al., 1985; Ebel and Grisebach, 1988). A well investigated plant-pathogen system is root and stem rot of soybean caused by *Phytophthora megasperma* f. sp. *glycinea*. Two lines of evidence indicate that glyceollin I is an important early defensive barrier erected by the plant against the invading pathogen and involved in determination of cultivar/race specific compatibility. Firstly, a detailed analysis of the distribution of fungal hyphae in plant tissue and the spatial and temporal pattern of glyceollin I accumulation was performed by Hahn et al. (1985). They found that limitation of fungal growth in the incompatible interaction is correlated with a rapid accumulation of glyceollin I at the infection site. Secondly, treatments that reduce the amount of phytoalexin present in resistant plants cause a corresponding increase in sensitivity to pathogens. For example, Waldmüller and Grisebach (1987) found that inhibition of glyceollin production with the PAL inhibitor APEP enables a normally incompatible race of *Phytophthora megasperma* f.sp. *glycinea* to infect soybean roots. By using indirect immunofluorescence of hyphae in cryotome cross-sections of roots, they could show that the growth pattern of the incompatible race 1 of *Phytophthora megasperma* f. sp. *glycinea* changed to that of the compatible race 3 under conditions where APEP caused loss of resistance against *Phytophthora megasperma* f. sp. *glycinea* race 1.

In contrast to their well documented function in plant defense, less attention has been paid to another aspect of flavonoid physiology, namely their possible role in plant development. Flavonoids can function as modulators of polar auxin transport (Jacobs and Rubery, 1988). The isoflavonoid glyceollin acts at low concentrations synergistically with auxin to stimulate adventitious root formation on mung bean (Yoshikawa et al., 1986). Root primordia formation is also stimulated. Therefore, glyceollin appears to enhance early processes of root initiation rather than simply promoting development of already existing primordia. It is tempting to speculate that the induction of root formation by glyceollin is due to the ability of flavonoids to influence lateral auxin transport. Recently, Hirsch et al. (1990) found that certain flavonoids elicit the formation of nodule-like structures on alfalfa roots, an effect that was also found with synthetic auxin transport inhibitors (Hirsch et al., 1989).

Taken together, these findings suggest that flavonoids might be involved in meristem induction in both developmental processes, nodule and lateral root morphogenesis. If this is true, then one should expect an alteration of local flavonoid concentration preceeding meristem formation. It has already been shown that the flavonoid composition of root hairs undergoes changes during the symbiotic infection process (Parniske et al., 1988). In this paper we restricted our measurement to glyceollin I concentrations in root hairs and root exudate and report a significant glyceollin I induction by *Bradyrhizobium japonicum* 110*spc*4. A possible physiological role for the symbiotic glyceollin I production is discussed.

Materials and Methods

Chemicals

The PAL inhibitor (R)-(1-amino-2-phenylethyl)-phosphonic acid a generous gift of Dr. E. K. Baylis, Ciba-Geigy, Manchester, USA. The antiserum against glyceollin I was a gift from Prof. Dr. H. Grisebach, Freiburg, Germany. Anti-rabbit-solid-phase was obtained from Fa. Laboserv, Gießen, Germany. Perlite was obtained from the Deutsche Perlite GmbH, Dortmund, Germany and Vermiculite from the Deutsche Vermiculite Dämmstoff GmbH, Sprockhövel (Haßlinghausen), Germany.

Growth of seedlings

Soybean seeds (*Glycine max* cv. Preston, Pioneer Hi-Bred Intern. Inc., Iowa, USA, *Glycine max* cv. Maple Arrow, Agriculture Canada Forage section, Ottawa, Ontario, Canada, *Glycine max* cv. Kenwood, Iowa State University, Ames, USA) were surface sterilized for 10 min in 30% H_2O_2, washed 7–10 times with sterile H_2O and soaked for 6–8 hours in sterile H_2O. Seedlings were grown in Vermiculite/Perlite (1 : 1) for 2 days at 25 °C, 13 Wm^{-2}, 75% humidity and a day night regime of 14 : 10 h.

Bacteria and culture condition

Bradyrhizobium japonicum 110*spc*4 (Regensburger and Hennecke, 1983), *Rhizobium meliloti* 2011 (Casse et al., 1979), *Rhizobium leguminosarum* PRE 8 (Dr. Lie, Agricultural University of Wageningen, Netherlands) and *Sinorhizobium fredii* HH 103 (Dowdle and Bohlool, 1985) were grown at 28 °C on a rotary shaker in 20E-Medium (Werner et al., 1975) or xylose minimal medium (XMM), a variation of the medium described by Tully (1985), containing 10 mM xylose as sole C-source and the following vitamins: 2×10^{-6} M 4-aminobenzoic acid, 5×10^{-6} M pyridoxine HCl, 1×10^{-4} M mesoinositol, 2×10^{-5} thiamine-di-HCl, 2×10^{-6} M Ca-D-pantothenate, 3×10^{-6} M D(+)-biotin, 1×10^{-5} M nicotinic acid.

Bacterial inoculum

Bacteria, grown in appropriate medium to mid log phase (max. 10^9 cfu ml^{-1}), were centrifuged and resuspended in 5 mM MES (pH 6.2) plus 1 mM $CaCl_2$ and adjusted to the desired cell densities. Bacteria were killed by autoclaving a buffer washed suspension (8×10^{10} cfu ml^{-1}) for 15 min at 120 °C. This treatment resulted in 100% killing as confirmed by plating aliquots on 20E agar plates. A cell free bacterial exudate was obtained from a cell suspension of *Bradyrhizobium japonicum* in buffer (8×10^{10} cfu ml^{-1}), stirred at room temperature for 1 h. Cells were removed by centrifugation and the supernatant was filtered through a 0.2 μm cellulose-nitrate-filter. Crude cell extracts of rhizobia grown in

XMM were prepared using a French press. Cell debris were removed by centrifugation. The extracts were adjusted to an equivalent concentration of 2×10^{10} cfu ml^{-1} with MES buffer.

Culture and zoospore induction of Phytophthora megasperma *f. sp.* glycinea

Phytophthora megasperma f. sp. *glycinea* race 1 was obtained from T. Waldmüller and J. Ebel (Freiburg) and cultured on Lima-Bean-Agar (Difco) in petri dishes at 25 °C in the dark. Zoospores were obtained from 6 day old cultures according to Eye et al. (1978) with the exception that agar plates with *Phytophthora megasperma* f. sp. *glycinea* mycelia were washed with mineral solution (0.58 g l^{-1} $Ca(NO_3)_2$, 0.15 g l^{-1} $MgSO_4 \times 7\ H_2O$, 1.15 g l^{-1} KH_2PO_4, 0.06 g l^{-1} KCl) instead of distilled water.

Inoculation procedure

Roots of 2 day old soybean seedlings were transferred to 2.3 ml test tubes containing either buffer, the respective bacteria-derived inducing agents or a suspension of *Phytophthora megasperma* f. sp. *glycinea* zoospores (2.5×10^4/plant). After an incubation time of 20 h (except where otherwise indicated) under the conditions described above, the volume of the liquid in the test tubes was made up to 2.3 ml with buffer (compensation for different transpiratory losses) and aliquots were removed for quantification of glyceollin I. The root hairs were harvested by scraping them with a sharpened spatula into an Eppendorf-cup.

Quantification of Glyceollin I

Freeze-dried root hairs were extracted with 1 ml methanol, 0.5 ml of the extract was evaporated to dryness and redissolved in 10% methanol. An aliquot of the root exudate was adjusted to 10% methanol. Glyceollin I concentrations in root hair extracts and root exudate were determined by a modification of the radioimmunoassay of Hahn et al. (1985), in which anti-rabbit-solid-phase was used to precipitate Anti-Glyceollin I. Appropriate dilutions were assayed in duplicate.

Application of PAL inhibitor APEP during seedling infection

The effect of the PAL inhibitor APEP on nodule formation was assayed on plants growing in growth pouches (Northrup, King Seed Co., Minneapolis, MN, USA). Examples and detailed descriptions of applications of the growth pouch technique for the analysis of early events in the soybean-*Bradyrhizobium japonicum* symbiosis are given by Bhuwanesvari et al. (1981) and Caetano-Anollés et al. (1991). Two-day-old seedlings of *Glycine max* cv. Preston were incubated for 20 h in 2.3 ml test tubes containing a *Bradyrhizobium japonicum* 110*spc*4 suspension (5.2×10^3 cfu/plant) in MES buffer and the inhibitor at different concentrations. Subsequently, the seedlings were transferred into sterile growth pouches and the position of the root tip was marked with a felt-tip pen on the transparent pouch. This root tip mark represents the time point at which the roots were removed from the inhibitor solution. When necessary, growth pouches were wetted with sterile half concentrated nutrient solution (Werner et al., 1975). After 11 days of growth under controlled environmental conditions (Werner et al., 1975), position and number of the nodules were determined.

Results

Bradyrhizobium japonicum *110spc4 induced production of glyceollin I*

Effect of inoculum cell density on glyceollin I production

The initial studies focussed on the effect of *Bradyrhizobium japonicum* on phytoalexin concentration in roots and root exudate of *Glycine max*. When buffer-washed cells of *Bradyrhizobium japonicum* were applied to soybean roots, we observed that glyceollin I accumulated during the symbiotic interaction. Phytoalexin levels in root exudate increased with increasing infection titers (Fig. 1). Beginning with inoculation titers of 10^{10} cfu/ml, *Bradyrhizobium japonicum* induced significant amounts of glyceollin I in the root exudate which were up to 50 fold higher than those of control seedlings (Fig. 1), whereas no glyceollin I accumulated in root hairs.

To exclude the possibility that the induction was caused by traces of medium-derived yeast extract which might contain fungal elicitors of phytoalexin accumulation, we incubated the seedlings with bacteria grown in yeast-free xylose minimal medium. These bacteria also induced elevated glyceollin I production. The effects were independent of the bacterial growth phase (data not shown).

Kinetics of glyceollin I accumulation during symbiotic interaction

The kinetics of glyceollin I accumulation in response to inoculation with *Bradyrhizobium japonicum* is shown in Fig. 2. At every time point analysed, the major proportion of the glyceollin I produced appeared in the root exudate (Fig. 2). Maximum glyceollin I levels were reached after 10 h of incubation and this level was maintained for up to 43 h.

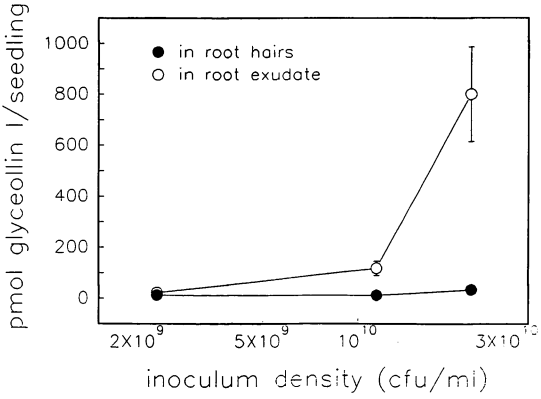

Fig. 1 Glyceollin I (pmol/seedling) in root hairs and root exudate of *Glycine max* cv. Preston after 20 h of incubation in cell suspensions of *Bradyrhizobium japonicum* 110*spc*4 grown in 20E medium, washed and resuspended in MES buffer. Each point represents the mean of data from 7–10 seedlings. The MES control seedlings contained on average 20 pmol glyceollin I/seedling in root hairs and 22 pmol glyceollin I/seedling in the root exudate. Bars indicate SEM values.

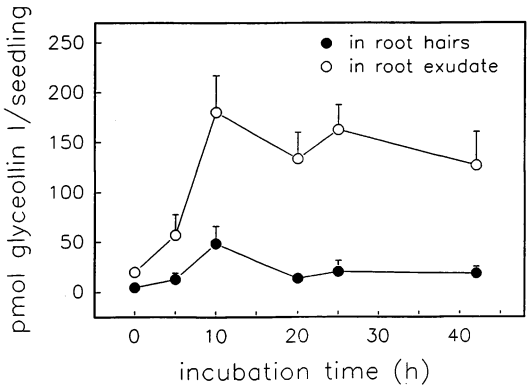

Fig. 2 Glyceollin I (pmol/seedling) in root hairs and root exudate of *Glycine max* cv. Preston at different times after exposure to a suspension of 3.9×10^{10} cfu/ml *Bradyrhizobium japonicum* 110*spc*4 cells, grown in 20E medium, washed and resuspended in MES buffer. Each point represents the mean of data from 8 seedlings. The MES control seedlings contained on average 5 pmol glyceollin I/seedling in root hairs and 20 pmol glyceollin I/seedling in the root exudate. Bars indicate SEM values.

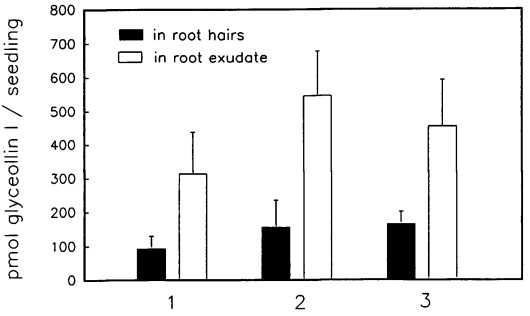

Fig. 3 Glyceollin I (pmol/seedling) in root hairs and root exudate of *Glycine max* cv. Preston after 20 h of incubation in different preparations of a *Bradyrhizobium japonicum* 110*spc*4 cell suspension. 1 = Bacteria grown in 20E medium, washed and adjusted to 8×10^{10} cfu/ml with MES buffer. 2 = same as 1, but cells were autoclaved for 15 min prior to application to the seedlings. 3 = same as 1, but cells were removed by centrifugation and the supernatant, filtered through a 0.2 μm pore size filter, was used for the plant assay. Each column represents the mean of data from 8–10 seedlings. The MES control seedlings contained on average 3 pmol glyceollin I/seedling in root hairs and 10 pmol glyceollin I/seedling in the root exudate. Bars indicate SEM values.

Glyceollin I induction by living cells, dead cells and cell-free exudate of *Bradyrhizobium japonicum* 110*spc*4

Glyceollin I production by soybean roots after incubation with *Bradyrhizobium japonicum* might be due to the infection of epidermal root cells by rhizobia. Alternatively, it may be a response to bacterial elicitors, independent of root hair penetration. In order to assess the probability of these alternatives, we tested dead bacteria, which are unable to infect the root, for their inducing capability.

As shown in Fig. 3, autoclaved rhizobia induced a glyceollin I response that was fully comparable with that obtained by living bacteria. Both the magnitude of the response as well as the distribution pattern of phytoalexin between root hairs and exudate were similar. Obviously, the plant reaction seems to be independent of the presence of living bacteria. Based on this observation we concluded that a penetration of the root surface is not necessary for the induction of glyceollin I accumulation.

During the incubation time of the plant assay, the dead bacteria sedimented to the bottom of the test tubes, leaving the roots submerged in a clear supernatant. It was therefore suspected that a soluble factor might be sufficient for the induction of phytoalexin accumulation. In order to test this hypothesis, we repeated the experiment with cell-free bacterial exudate. As shown in Fig. 3, the bacterial exudate induced a similar reaction as did living or dead cells. This result shows that the elicitor of the plant reaction is a soluble factor $< 0.2\ \mu$m.

Glyceollin I production by soybean roots during phytopathogenic interactions

Symbiotic and pathogenic interactions were compared with respect to glyceollin I accumulation which they induced in the rhizosphere of soybean. Since there are no well characterised soybean root pathogenic bacterial strains available, zoospores of *Phytophthora megasperma* f. sp. *glycinea*, a soybean root pathogenic oomycete, were tested for their effect on glyceollin I production by soybean roots. This oomycete has commonly

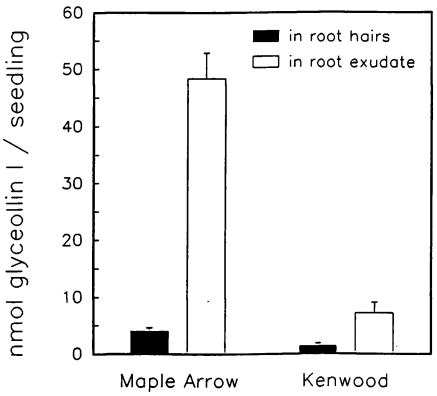

Fig. 4 Glyceollin I (nmol/seedling) in root hairs and root exudate of an incompatible (Maple Arrow) and a compatible (Kenwood) *Glycine max* cultivar after 20 h of incubation with 2.5×10^4 zoospores of *Phytophthora megasperma* f. sp. *glycinea* race 1/seedling. Each column represents the mean of 10 seedlings. Bars indicate SEM values.

been used to investigate phytoalexin responses of soybean roots and it is well established that *Phytophthora megasperma* f. sp. *glycinea* infection induces high concentrations of glyceollin I in root tissue (Hahn et al., 1985). In addition, our data show that glyceollin I accumulates in the root exudate (rhizosphere) of such seedlings. As illustrated in Fig. 4, a seedling of the cultivar Kenwood (compatible with *Phytophthora megasperma* f. sp. *glycinea*) exuded about 7 nmol glyceollin I during the first 20 h of a successful infection with *Phytophthora megasperma* f. sp. *glycinea* race 1. Roots of these seedlings became brown, lost turgidity and developed no secondary roots. At later stages, hyphae could often be observed growing out of the cotyledons. In contrast, the incompatible interaction with seedlings of cultivar Maple Arrow was characterized by a darkening of the root tip and the development of numerous secondary roots. The shoots could not be distinguished from those of buffer-treated plants. Those seedlings, which were able to resist pathogenic attack, produced about 48 nmol glyceollin I/seedling during the first 20 h after infection with zoospores of *Phytophthora megasperma* f.sp. *glycinea* race 1. These glyceollin I concentrations in the root exudate are considerably higher than those induced by all other treatments applied in this study.

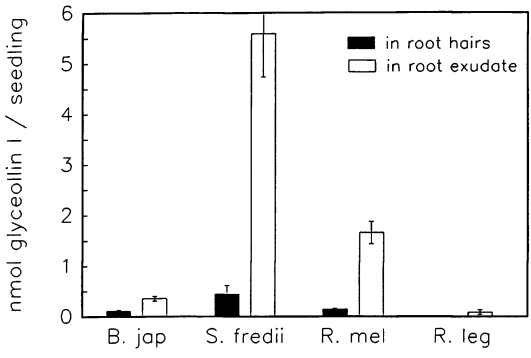

Fig. 5 Glyceollin I (nmol/seedling) in root hairs and root exudate of *Glycine max* cv. Kenwood after 20 h of incubation with cell extracts of different rhizobia. Cells were grown in XMM and broken using a French Press. Cell debris were removed by centrifugation and the resulting extracts were adjusted to an equivalent concentration of 2×10^{10} cfu/ml. Each column represents the mean of data from 7–9 seedlings. The MES control seedlings contained on average 8 pmol glyceollin I/seedling in root hairs and 24 pmol glyceollin I/seedling in the root exudate. Bars indicate SEM values.

Glyceollin I induction by rhizobial strains

Bradyrhizobium japonicum induces the production of low but significant amounts of glyceollin I by *Glycine max*. These glyceollin I levels remain low even at high inoculum densities, indicating that the microsymbiont is capable of suppressing or preventing this plant defense response. Such a reduction in the plant defense response could be a prerequisite for a successful infection. We were interested whether rhizobia that are unable to nodulate soybean differ from *Bradyrhizobium japonicum* with respect to the amounts of glyceollin I/seedling they induce. When soybean roots were infected with suspensions of *Rhizobium meliloti* (unable to nodulate *Glycine max*), highly variable responses were observed. These ranged from no response up to a production of 3 nmol/seedling in the root hairs. It was determined that the variation was not due to the length of the incubation time (data not shown). However, the glyceollin I concentration seemed to be dependent on the batch of bacterial cells used. In order to analyse the factors responsible for the variation, we tested crude cell extracts for their inducing capacity. Interestingly, incubation in such a preparation of *Rhizobium meliloti* cells always led to very high glyceollin I concentrations in the nmol-range.

Since it was found that the use of cell extracts led to more reproducible reactions by plant roots, these conditions were chosen to test two additional rhizobial strains (Fig. 5). *Sinorhizobium fredii*, another *Glycine max* nodulating bacterium, and *Rhizobium leguminosarum*, a bacterium which is unable to nodulate *Glycine max*, were used. The two strains tested that are unable to nodulate soybean, induced different glyceollin I levels. The glyceollin I levels increased significantly after incubation with the supernatant from a crude *Rhizobium meliloti* cell extract, whereas no enhancement could be observed after incubation with a comparable preparation from *Rhizobium leguminosarum* cells. In addition, there were strong differences between the two soybean nodulating rhizobia analysed in this study. Disrupted *Bradyrhizobium japonicum* cells failed to induce any more glyceollin I accumulation than whole cells (compare data from Fig. 5 with Figs. 1, 2, 3). As seen in Fig. 5, a crude extract of *Sinorhizobium fredii* cells induced glyceollin I concentrations in soybean root exudate which are in the same order of magnitude as those found after compatible infection with zoospores of the root pathogen *Phytophtora megasperma* f. sp. *glycinea* race 1.

No correlation could be found between the ability of the rhizobial strains to infect soybeans and the glyceollin I levels induced by cell preparations from these strains.

Absolute glyceollin I concentrations showed considerable variations between different tests i.e. different batches of seedlings. This variation in phytoalexin response between different batches of seedlings was also observed by other workers (e.g. Bonhoff and Grisebach, 1988; Hahn et al., 1985). Consequently each figure contains data derived from the same test.

Inhibition of nodulation by the PAL-inhibitor APEP

Having observed low but significant induction of the phytoalexin glyceollin I after infection with *Bradyrhizobium japonicum*, we were interested to assess the physiological significance of this in the early stages of the symbiotic interaction. Our approach was to analyse the effect of an inhibitor of flavonoid synthesis on nodule formation.

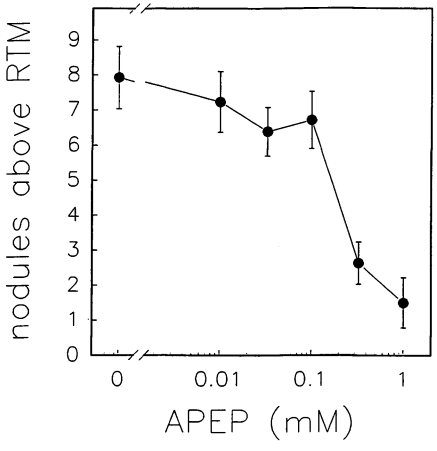

Fig. 6 Effect of the PAL inhibitor APEP on the number of nodules formed above the root tip mark after infection of *Glycine max* cv. Preston with *Bradyrhizobium japonicum* 110*spc*4 (5.2×10^3 cfu/plant). The primary roots were incubated for 20 h in 2.3 ml test tubes containing inhibitor and bacteria, then transferred to growth pouches. The position of the root tip was marked. After 11 days, position and number of the nodules were determined. Each point represents the mean of data from 12–16 plants. Bars indicate SEM values.

Table 1 Effect of preculture of *Bradyrhizobium japonicum* 110*spc*4 in isoflavonoid-containing medium on the inhibition of nodulation by APEP.

Preculture[1] of bacteria	Nodule number[2] above rtm after incubation of roots in MES	APEP
control	8.5[a]	1.5[b]
isoflavonoid	6.5[a]	1.0[b]

[1] *Bradyrhizobium japonicum* was precultured in 20E medium containing either 10 μM each of daidzein and coumestrol (isoflavonoid) or 0.02 % (v/v) dimethylformamide (control). Plants were infected with 8×10^3 cfu/plant. All other conditions were the same as described in Fig. 6.

[2] Values with different indices differ with a significance of < 5 % (t-test).

A key enzyme in flavonoid synthesis is the phenylalanine-ammonia-lyase (PAL). A specific inhibitor of this enzyme is (R)-(1-amino-2-phenylethyl)phosphonic acid (APEP) (Laber et al., 1986). The effect of the PAL inhibitor on nodulation was tested with seedlings growing in growth pouches. In this experiment, the position of the root tip mark (rtm) corresponded to the time of removal of the root from the inhibition solution. Starting with APEP concentrations of 330 μM, the number of nodules over the rtm was found to be drastically reduced (Fig. 6). On APEP treated plants, a burst of nodulation was typically observed directly below the rtm (data not shown) indicating that nodulation was possible as soon as the inhibitor was removed. The highest APEP concentration applied in this experiment (1 mM) inhibits neither bacterial nor plant growth (data not shown). These observations show that the reduced nodule initiation in the presence of APEP is not due to a non-specific toxic effect. The nodulation of APEP-treated seedlings could not be increased by using rhizobia that had been previously grown in a medium containing coumestrol and daidzein, the major isoflavonoid constituents in root exudate of soybean seedlings (D'Arcy-Lameta, 1986) (Table 1). These results suggest that the reduced nodule number in root regions that have been in contact with the inhibitor results from a specific inhibition of a metabolic function which is indispensable in the early symbiotic interaction.

Discussion

In this study, glyceollin I production by single seedlings of *Glycine max* in symbiotic and pathogenic interactions was analysed. Axenically grown seedlings produce very low amounts of glyceollin I in root hairs as well as in root exudate. In contrast, when seedlings were incubated with living cells of *Bradyrhizobium japonicum* 110*spc*4, a bacterium that is able to form effective nodules on *Glycine max* roots, a significant production of glyceollin I was observed. Typically, most of the glyceollin I produced during the symbiotic interaction accumulated in the root exudate, whereas a smaller portion was found in root hairs. The elicitor of this reaction appears to be a soluble, heat stable factor of a size < 0.2 μm since autoclaved cells as well as filtered cell-free bacterial exudate or a crude cell extract had the same inducing capacity. The maximum glyceollin I accumulation was reached after 10 h. The concentration of glyceollin I in root exudate did not increase during longer incubation.

For the pathogenic interaction between *Glycine max* and *Phytophthora megasperma* f. sp. *glycinea*, evidence has accumulated that the production of glyceollin plays an important role in resistance of the host plant towards incompatible races of *Phytophthora megasperma* f. sp. *glycinea* (Waldmüller and Grisebach, 1987; Hahn et al., 1985). Our data confirm this idea since during the first 20 h of the incompatible interaction (*Glycine max* cv. Maple Arrow – *Phytophthora megasperma* f. sp. *glycinea* race 1), much higher amounts of phytoalexins accumulated than during the compatible interaction (*Glycine max* cv. Kenwood-*Phytophthora megasperma* f. sp. *glycinea* race 1).

In contrast to pathogenic systems, where incompatibility is correlated with glyceollin I levels, no connection could be found between the ability of a rhizobial strain to form nodules on *Glycine max* and the amount of glyceollin I produced in root hairs or exudate. Unexpectedly, the highest glyceollin I levels, induced by crude bacterial cell extract, were obtained with *Sinorhizobium fredii* (able to nodulate *Glycine max*), whereas the lowest levels were obtained with *Rhizobium leguminosarum* (unable to nodulate *Glycine max*) (Fig. 5). However, there was a strong variation observed amongst the strains that were unable to nodulate *Glycine max*, since *Rhizobium meliloti* induced higher levels of glyceollin I than *Rhizobium leguminosarum*. The comparison of the four rhizobial strains, with respect to their glyceollin I inducing capacity, showed no correlation between their ability to infect *Glycine max* and the glyceollin I levels induced by crude cell extracts from these strains. Certainly, these conclusions have to be prefaced with the remark that these results were obtained by the use of crude cell extracts since

it was impossible to obtain reproducible plant reactions with living *Rhizobium meliloti* cells. It is possible that these conditions do not reflect the *in vivo* situation.

In this study we observed that *Bradyrhizobium japonicum* 110*spc*4 induces a small but significant glyceollin I exudation by *Glycine max* roots. Estabrook and Sengupta-Gopalan (1991) analysed mRNA levels for PAL and CHS in roots of *Glycine max* after symbiotic infection with *Bradyrhizobium japonicum* USDA 110. They used these key enzymes of the phenylpropanoid and flavonoid biosynthetic pathway to investigate if an induction of the pathway, leading to symbiotic signal molecules and phytoalexins like glyceollin I, had taken place and were able to show significant increases in the quantity of specific mRNAs even on the first day after inoculation. This finding is consistent with the glyceollin I exudation observed in our study.

Flavonoid accumulation in the root exudate of leguminous plants in response to symbiotic infection was also described by Recourt et al. (1991). They reported that inoculation with the homologous symbiont *Rhizobium leguminosarum* bv. *viciae* results in an increase of *nod* gene inducing flavonoids in root exudate of *Vicia sativa* subsp. *nigra*.

The increased glyceollin I levels, induced by the symbiotic microorganism, are in line with the previously observed increase in flavonoid synthesis (Parniske et al., 1988). Nevertheless, the physiological significance of glyceollin I exudation by soybean roots is still unclear. An increase in phenylpropanoid derivative concentration seems to be indispensible for a successful symbiotic interaction since its inhibition leads to a significant reduction in nodule formation. That PAL inhibition in alfalfa roots leads to a reduced synthesis and release of flavonoids was reported by Maxwell and Phillips (1990). We observed that inhibition of flavonoid and glyceollin I synthesis with the PAL inhibitor APEP, drastically reduced induction of nodule formation. The negative effect of PAL inhibition on nodule formation could not be explained by an absence of *nod* gene inducers since nodule formation was still inhibited when the bacteria were grown in an isoflavonoid-(= *nod* gene inducers) containing medium (Tab. 1). The effective inhibitor concentrations were the same as described by Waldmüller and Grisebach (1987). They used a similar assay design and observed a 46% inhibition of glyceollin I production with 330 μM APEP and a 67% reduction with 1 mM APEP. Based on their data, we conclude that PAL and glyceollin I synthesis were also inhibited in our experiment. The reduction in nodule formation caused by APEP suggests that flavonoid compounds, induced after symbiotic infection, are important mediators of early symbiotic events.

Some recent results from several independent laboratories indicate a possible role of flavonoids in meristem induction. For the induction of root formation, local concentrations of plant hormones, like auxin and cytokinin, have been suggested to be critical parameters (Libbenga et al., 1973). It was shown by Jacobs and Rubery (1988) that certain flavonoids can specifically compete with a synthetic auxin transport inhibitor for binding to its receptor. These flavonoids disturb polar auxin transport in a manner similar to that of synthetic auxin transport inhibitors and therefore may have an influence on auxin distribution and local concentrations. In that connection it is an interesting finding that enhanced expression of a bean CHS8-GUS gene fusion was found in root areas of transgenic tobacco where the formation of lateral roots had been initiated (Schmid et al., 1990). CHS is a key enzyme of flavonoid synthesis.

Since both the initiation of lateral roots and that of symbiotic root nodules (Rolfe and Gresshoff, 1988) require the induction of meristematic activity, the occurrence of similar physiological processes during the ontogenesis of the two root-derived plant organs seems possible. The close ontogenic relationship between nodules and lateral roots is also evidenced by the observation that some *Rhizobium meliloti* strains stimulate both normal nodules and "hybrid" structures intermediate between a nodule and a lateral root (Dudley et al., 1987). The finding that auxin transport inhibitors, as well as certain flavonoids, elicit nodule-like structures on alfalfa, which closely resemble *Rhizobium*-induced legume nodules (Hirsch et al., 1989, 1990), is evidence for the critical role of auxin during nodule development.

The glyceollin I levels induced in soybean roots infected with the compatible microsymbiont *Bradyrhizobium japonicum* 110*spc*4 were low when compared to those characteristic of a pathogenic interaction. In both types of pathogenic interactions, compatible and incompatible, the levels of glyceollin I production were several times higher than those observed as a result of the symbiotic interaction. It is likely that at these low concentrations glyceollin I has plant hormone characteristics and plays a role in nodule induction rather than in plant defense. Evidence for glyceollin I as a plant hormone comes from the experiments of Yoshikawa et al. (1986) who observed an increased meristem induction by mung bean roots in response to glyceollin I/auxin treatments. Our results indicate that the plant is in need of PAL products during the early stages of nodule formation. Experiments are in progress to analyse whether this is due to the involvement of flavonoids in meristem induction.

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