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Legume Root Metabolites and VA-Mycorrhiza Development

RÜDIGER KAPE, KERSTIN WEX, MARTIN PARNISKE, ELISABETH GÖRGE, ASTRID WETZEL, and DIETRICH WERNER

Fachbereich Biologie, Botanisches Institut der Philipps-Universität Marburg, Karl-von-Frisch-Straße, D-3550 Marburg, Germany

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Summary

The communication by phenylpropane-metabolites between symbiotic microorganisms and their legume host plants was further studied by the elucidation of root segment-specific exudation of aromatic compounds. The excreted flavonoids were collected by blotting seedling roots directly onto cellulose acetate filters. The flavonoids were eluted from filter segments and subsequently analysed by capillary electrophoresis.

Effects of various flavonoids on VA-mycorrhiza (VAM) development were studied. *Glomus mosseae* and *Glomus intraradix* (Schenck and Perez, 1990) were used in these studies. Daidzein (2 to 5 µM) increased germination of spores in a period between 15 to 30 days. Myricetin increased hyphal growth of germinated spores at a concentration of 2 µM between 20 and 50 days. Quercetin had a similar effect in a concentration range of 0.8 to 2.0 µM, whereas 5 µM slightly inhibited the growth of hyphae from the germinating spores.

Phytoalexin production in VA-mycorrhiza infected legume roots was studied with *Vicia faba* by wyerone concentration to quantify the reaction of a non-aromatic secondary root metabolite. VAM infected roots increased wyerone concentration by a factor of 3 to 5 compared with the control, however, at a very low absolute level.

A phenylpropane-communication concept for *Rhizobium* infected legumes is discussed in relation to a still incomplete similar scheme for VAM.

Key words: Flavonoids, wyerone, root development, VA-mycorrhiza.

Abbreviations: MES = 2-(N-morpholino)ethane sulfonic acid; MGD = 6″-O-malonyl-7-O-β-D-glucosyldaidzein; VAM = vesicular-arbuscular mycorrhiza.

Introduction

On the basis of chemotaxis, *nod* gene inducing activity (Kape et al., 1991), phytoalexin production (Parniske et al., 1991 a; Schmidt et al., 1992) and isoflavonoid inducible resistance in rhizobia (Parniske et al., 1991 b), a new communication concept for the *Bradyrhizobium*-legume symbiosis was proposed. The basic of this concept is that metabolites of the phenylpropane pathway have very different functions: precursors such as cinnamic acid and p-coumaric acid have mainly a chemotactic effect, whereas intermediates such as isoliquiritigenin and daidzein have *nod* gene inducing activity as well as glyceollin resistance inducing activity. Endoproducts such as glycinol and glyceollin still have this function, but are not so efficient (Kape et al., 1992).

In the present publication our aim was to contribute to a further development of the concept in two directions: the identification of root segment-specific aromatic compounds with signalling functions and the study of the effect of some of the identified compounds on the first stages of VA-mycorrhiza development (spore germination and hyphal growth from spores). Some data on VA-mycorrhiza development in the legume species used are also included.

Materials and Methods

Production of soybean seed and root exudate

The production of soybean (*Glycine max* (L.) Merr. cv. Maple Arrow) root exudate is described by Kape et al. (1992). It made use of the ability of flavonoids to absorb to cellulose acetate filters (Recourt et al., 1991).

Soybean (cv. Maple Arrow) seed exudate was produced with a similar procedure. Seeds were sterilized as described in Kape et al. (1992). The seeds were subsequently placed on a sterile cellulose acetate filter and imbibed for 24 h in 2-(N-morpholino)ethane sulfonic acid buffer (5 mM, pH 6.2) plus 1 mM $CaCl_2$ (MES-$CaCl_2$) on the filtèr. The flavonoids were extracted from the filter and analysed by HPLC as recently described (Kape et al., 1992).

Analysis of local differences in the exudation of flavonoids along the root of soybean seedlings

Soybean seeds (cv. Preston) were surface sterilized for 10 min in 30 % hydrogen peroxide. After sterilization, seeds were washed thoroughly and imbibed for 2.5 h in sterile tap water. Afterwards, seeds were placed on agar (1.5 %) prepared with MES-$CaCl_2$. Seedlings were grown in a growth cabinet (25 °C; relative humidity, 75 %, 16–8 h light-dark period; irradiance 13 Wm^{-2}, white light).

The roots of 4-day-old seedlings were placed between two cellulose acetate filters moistened with MES-$CaCl_2$. Layers of moist tissue papers were used to wrap the seedlings plus the filters. The moist tissue papers assured close contact between the seedling root and the filters, and prevented desiccation of the seedlings. After 1 hour the seedlings were removed and the filters dried at room temperature.

Both filters originating from one seedling root were cut into sections of 4 mm. Flavonoids were extracted with methanol from the corresponding filter sections belonging to identical segments of a root.

The methanolic solution was dried in a stream of nitrogen at room temperature. The residue was dissolved in 100 µL electrophoresis buffer (50 mM boric acid, 10 mM sodium tetraborate, pH 8.5). The samples were analysed with capillary zone electrophoresis (Dionex CES 1, Sunnyvale, California/USA) supplied with a fused silica column (inside diameter 75 µm, length 60 cm); 45 nL of the sample was loaded into the column. The separation was performed at a voltage of 20,000 V with positive polarity for the sample. The electrophoresis buffer used for flavonoid separation was adjusted to pH 9. Compounds were detected with a variable-wavelength detector at 250 nm.

Flavonoids were identified by their retention time and by coelectrophoresis with authentic standards. Daidzein and genistein were purchased from Serva (Heidelberg, Germany), coumestrol from Kodak-Eastman (Rochester, USA).

VA-mycorrhiza-methods

Isolation of spores: Spores were isolated by wet-sieving according to Gerdemann and Nicolson (1963). The isolated spores were sterilized (modified from Mosse, 1959) in the following solution: 2 % (w/v) chloramine T, 0.02 % (w/v) streptomycine sulphate and 0.05 % (w/v) tween 40. For sterilization, the spores were placed on a paper filter and a permanent flow of sterilizing solution through the filter was applied for 20 min. Spores were then carefully rinsed several times with sterile tap water.

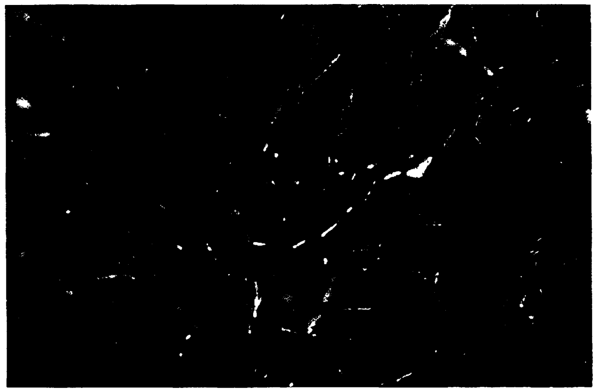

Fig. 1: Fluorescence photograph of soybean root exuded compounds absorbed to a cellulose acetate filter in UV light (254 nm). The fluorescent compounds were exuded by seeding roots of *Glycine max* cv. Maple Arrow growing on the surface of the filter. The arrow points at a side root emerging from the main root. The origin of the side root is characterized by increased fluorescence.

Axenic spore germination and hyphal growth from spores were tested on buffered water agar (MES 10 mM, 8.76 mM NaOH, 11.24 mM NaCl, pH 7.0).

To discriminate living from dead VA-mycorrhiza spores equal volumes of a spore suspension and a 0.05 % (w/v) nitroblue tetrazolium solution were mixed at room temperature and incubated for 40 h. Living spores produced a brilliant red colour, whereas dead spores were uncoloured or became blue after prolonged incubation periods.

Other methods

Cultivation and origin of *Rhizobium*, *Bradyrhizobium* and host plants (*Phaseolus vulgaris*, *Glycine max*, *Vicia faba*) were as previously described (Wolff et al., 1991; Buttery et al., 1990; Kinnback and Werner, 1991). For VA-mycorrhiza infection of legume host plants, the following preparations were used: 1. *Glomus intraradix* infected roots of *Allium schoenoprasum*, cut into 1 cm pieces, and 2. *Glomus mosseae* in a soil inoculum produced with *Helianthus annus* or *Allium* sp.

Both inocula were placed together with the plant seed into the growth substrate.

Wyerone (phytoalexin of faba beans) was isolated and analysed as described by Görge and Werner (1991).

Results and Discussion

Spatial pattern of flavonoid root exudation

Figure 1 visualizes the exudation of fluorescent phenolic compounds by roots of soybean (cv. Maple Arrow). The roots growing on the cellulose acetate filters leave traces of fluorescent phenolic compounds behind (e.g. flavonoids). Differences in the intensity of fluorescence in Fig. 1 may reflect some differences in the amounts and composition of the exuded compounds, although no quantitative interpretation is possible. Quantitative studies ascertain a close contact between the root and the cellulose acetate filter. Such a close

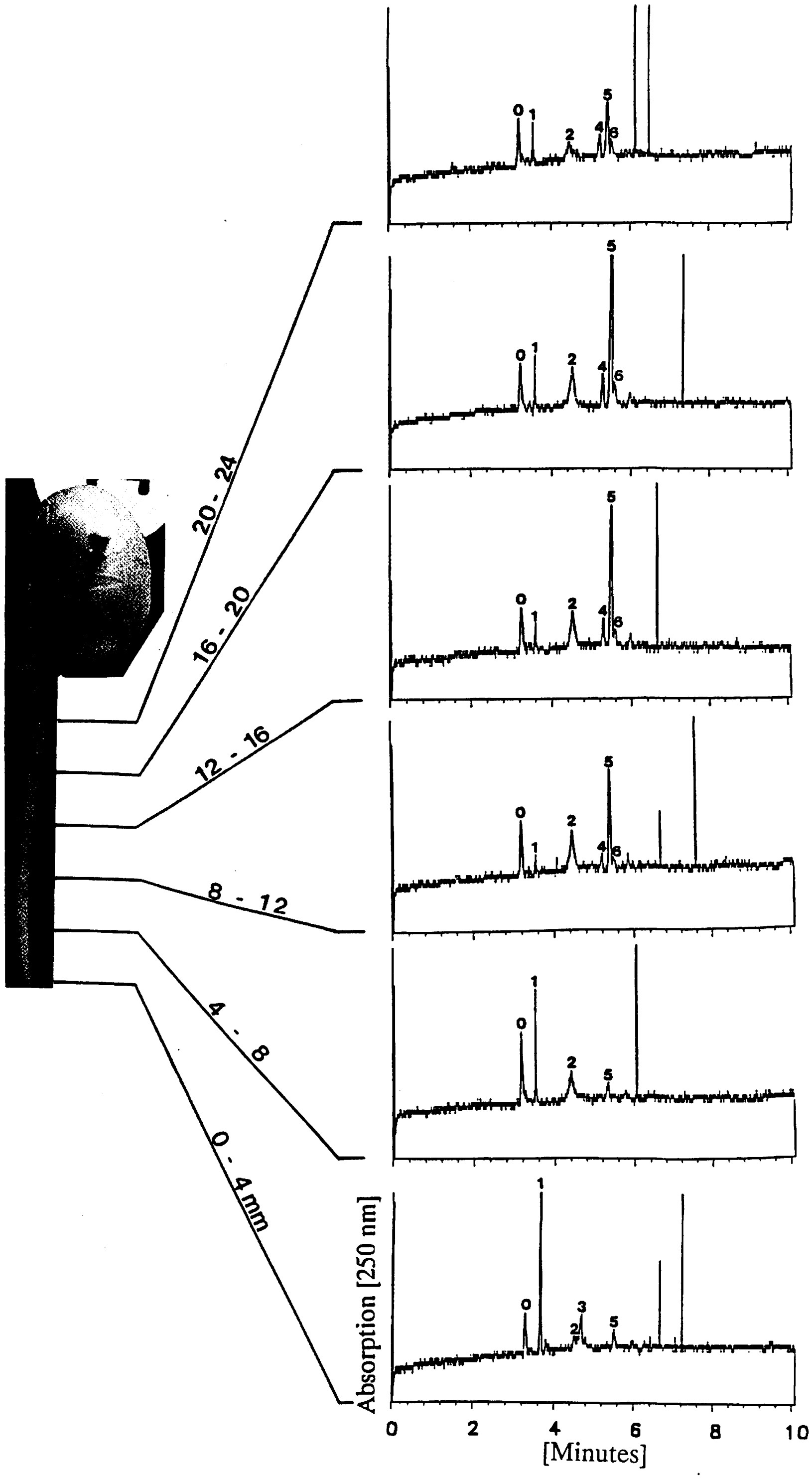

Fig. 2: Local differences in the root exudation of soybean seedlings. A 4-day-old seedling of *Glycine max* (cv. Preston) was used. The root exudates of individual segments (4 mm long) of this seedling were collected and analysed by capillary zone electrophoresis. The electropherograms of these exuded fractions are shown on the right side of the figure. UV absorbing compounds were detected at 250 nm. Peaks with identical retention times are labeled with the same numbers. Peak 5 was identified as daidzein and peak 6 as genistein (Peak O is an unknown contamination present in all samples).

contact was guaranteed in the analysis of the local root exudation. This quantitative analysis of flavonoids exuded by different soybean (cv. Preston) root areas is depicted in Fig. 2. The flavonoids exuded by different areas were analysed by capillary electrophoresis. The ratio of the five major compounds separated differs significantly in the root hair zone, the elongation zone and the root tip. Peak 5 was identified as daidzein, peak 6 as genistein. Especially the ratio of peak 1 (not identified) to daidzein (peak 5) changed to a large extent. Graham (1991) also found root segment specific flavonoid

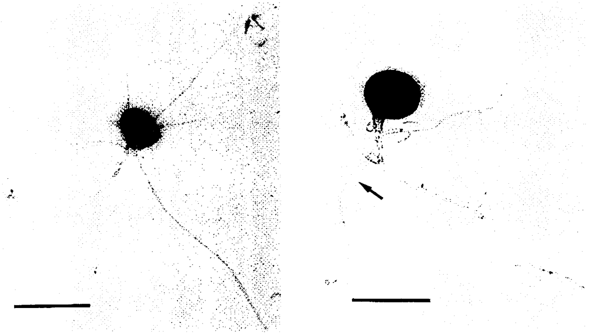

Fig. 3: Axenically germinated spores of *Glomus intraradix* (a) and *Glomus mosseae* (b) on water agar (bar, 125 μm). Only *G. mosseae*, but not *G. intraradix*, produces small vesicles (arrow).

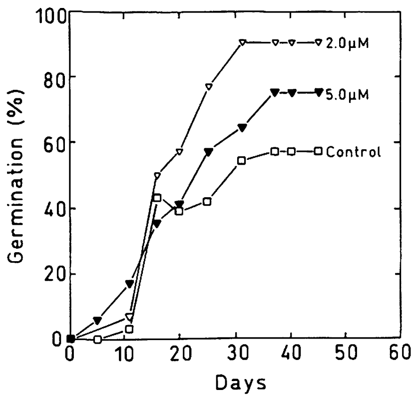

Fig. 4: Spore germination of *Glomus mosseae* on water agar with different concentrations of daidzein.

expression in soybean with another method of isolation of the root exudate. He found high concentrations of daidzein at the root tip and only small concentrations of MGD (6''-O-malonyl-7-O-β-D-glucosyldaidzein). Graham collected exudates with the help of cotton wicks. By heating the wick immediately after sampling, the ratio of daidzein to MGD changed significantly . This difference was explained by heat inactivation of β-glucosidase in the root exudate. In our method using a cellulose acetate filter, which was immediately dried after contact with the seedling and extracted by methanol, a possibly present β-glucosidase is unlikely to become active.

Differences between seed and root exudates

Differences in the flavonoid compositions of seed and root exudates of alfalfa were found by Phillips et al. (1990). The different flavonoids in the seed and root exudates had divergent effects on the microsymbiont of alfalfa, *Rhizobium meliloti* (Hartwig et al., 1990). To describe these effects on the microbial population in the areas around the seed and the root Phillips et al. (1990) used the term «ecochemical zones».

Also for soybean (cv. Maple Arrow), differences in the composition of seed and root exudates were found. Coumestrol and isoliquiritigenin were missing in the seed exudate, but were present in the root exudate (Kape et al., 1992). The isoflavonoids daidzein and genistein were found in different ratios in the root exudate (daidzein : genistein, 14 : 1) and in the seed exudate (3.2 : 1) as determined by HPLC analysis. In *B. japonicum*, the microsymbiont of *Glycine max*, these four flavonoids induce *nod* gene activity and glyceollin resistance to different extents. These data sup-

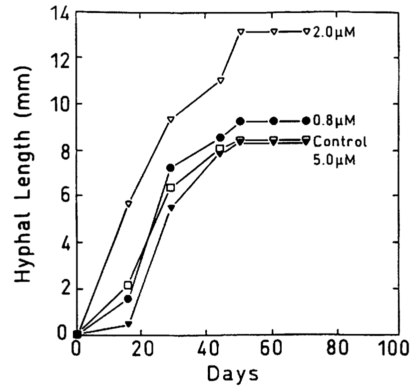

Fig. 5: Average hyphal length of *Glomus mosseae* per germinated spore on water agar with different concentrations of myricetin.

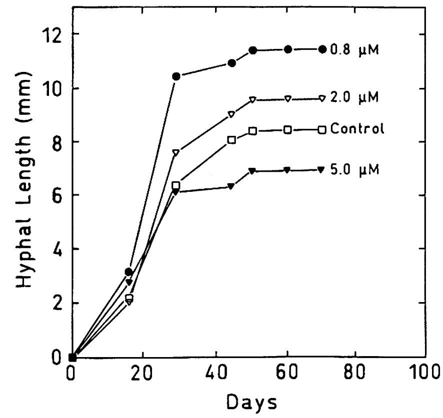

Fig. 6 a

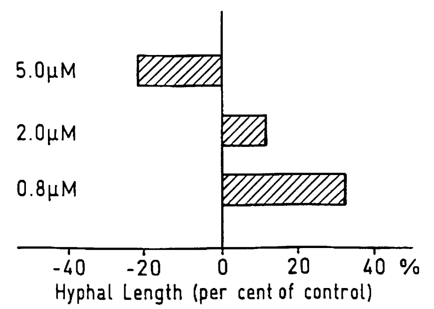

Fig. 6 b

Fig. 6: Effect of different concentrations of quercetin on hyphal growth of *Glomus mosseae*; (a) Time course of hyphal growth; (b) Hyphal growth as per cent of control without quercetin after 70 days.

port the concept of «ecochemical zones» postulated by Phillips et al. (1990).

Beneficial effects of flavonoids on VAM fungi development

The germination of spores of *Glomus mosseae* and *Glomus intraradix* without addition of flavonoids is documented in Fig. 3 (a, b). An interesting difference between the two species of *Glomus* is the production of small vesicles by *Glomus mosseae*, which are absent on the hyphae of *Glomus intraradix* on the germination medium used.

In the concentration range of 2.0 to 5.0 µM, daidzein stimulates spore germination (percentage of spores germinating) between day 15 and 30 (Fig. 4). The growth of the hyphae from germinating spores is stimulated by myricetin (2 µM) whereas lower and higher concentrations had no significant effect (Fig. 5). A rather similar result was found with quercetin (Fig. 6 a), with a maximal hyphal length of 11–13 mm per spore at a concentration of 0.8 µM. A concentration of

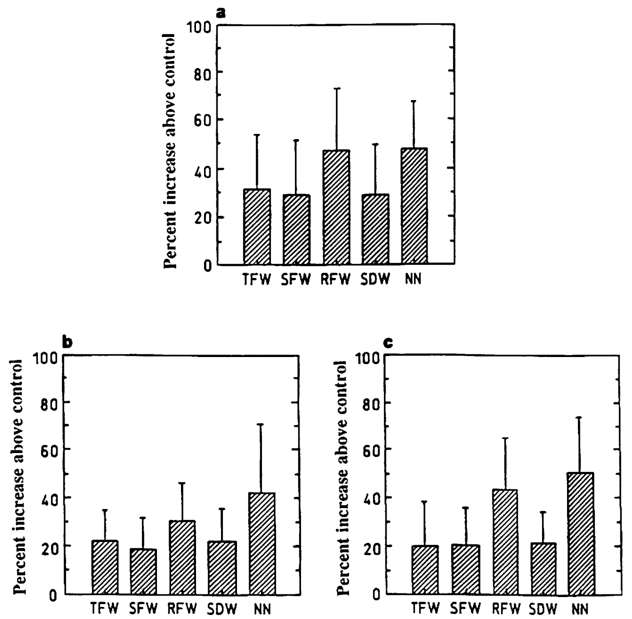

Fig. 8: Stimulation (percent increase above control) of the development of three different cultivars of *Phaseolus vulgaris* nodulated by *Rhizobium leguminosarum* bv. *phaseoli* through inoculation with *Glomus intraradix* after 8 weeks (a) Fori, (b) Saxa, (c) Hinrichs Riesen. Data are mean values of 14 plants. TFW = fresh weight of total plant, SFW = shoot fresh weight, RFW = root fresh weight, SDW = shoot dry weight, NN = nodule number.

Fig. 7: Structures of the VA-mycorrhizal fungus *Glomus intraradix* in the root cortex of *Phaseolus vulgaris* cv. *saxa.* Stained with trypan blue. (a) arbuscules (bar, 50 µm), (b) vesicles (bar, 50 µm), (c) spores (bar, 30 µm).

5 µM quercetin already had a significant inhibitory effect on hyphal growth (Fig. 6 b). After about 40 to 50 days hyphal growth stopped in all cases.

Also with other *Glomus* species and some other flavonoids, stimulation of hyphal growth was recently found. Apigenin (4′,5,7-trihydroxyflavone) stimulated germination and hyphal growth of *Gigaspora margarita* (Gianinazzi-Pearson et al., 1989). Liquiritigenin stimulated the germination of *Glomus etunicatum* by about 20 % (Tsai and Phillips, 1991) and Chrysin (5,7-dihydroxyflavone) the colonization of plant roots by an unidentified *Glomus* species at a concentration of 20 µM (Siqueira et al., 1991). Quercetin significantly (10 µM) prolonged hyphal growth from germinated spores of *Gigaspora margarita* and *Glomus etunicatum* (Bécard et al., 1992). In all cases so far, the mechanisms of the stimulation of germination and hyphal growth remain unexplained.

From a larger screening programme of combinations of various *Glomus* species with a number of legumes, we found the most reliable mycorrhization for the *Phaseolus vulgaris* cv. Saxa – *Glomus intraradix*-system (Fig. 7 a–c), where arbuscules, vesicles and spores were regularly formed at a high density. Root growth and *Rhizobium* nodule formation were more stimulated by VAM inoculation than stem and leaf growth (Fig. 8). These data were confirmed with two other cultivars of *Phaseolus vulgaris.*

Phytoalexin production after VA-mycorrhiza infection

To compare the production of aromatic compounds with non-aromatic secondary plant metabolites, we chose *Vicia*

Table 1: Wyerone concentrations in roots of *Vicia faba* with or without VA mycorrhiza (*Glomus aggregatum, Glomus macrocarpum* and *Glomus mosseae*) in dependence of the host cultivar 8 weeks after infection.

designation of cultivar	wyerone concentration (μg/g FW) without infection	with infection
Alfred	0.8	1.7
Kristall	0.4	3.2
TF	0.5	3.1

faba as a host plant. *Vicia faba* produces wyerone, the phytoalexin of faba beans (Mansfield and Hargreaves, 1974). With a mixture of three different *Glomus* species (*Glomus aggregatum, Glomus macrocarpum* and *Glomus mosseae*) we received the best mycorrhization. Using three different cultivars of *Vicia faba,* we found a wyerone concentration between 1.7 and 3.2 μg per g fresh weight in the roots (Table 1). The controls (without mycorrhization) were in the range of 0.4 to 0.8 μg per g fresh weight. This means that the roots react towards mycorrhiza infection with a detectable increase in this phytoalexin. Similar data were found by Morandi (Morandi et al., 1984; Morandi, 1990) with the phytoalexin glyceollin and soybean roots infected with VAM. These authors emphasized the differences between infected and noninfected roots. We interprete our data somewhat differently. We consider the very low concentration of 1.7–3.2 μg phytoalexin per g fresh weight as an indication of the almost perfect protection of the host root from the fungus by the VAM specific compartmentation. Similarly, we previously found very low glyceollin concentrations in wild-type combinations (fully effective) of *Rhizobium* and legume plants with 2 to 10 μg per g nodule fresh weight. However, in combination with symbiosis defective mutants of *Bradyrhizobium,* affecting the stability of the symbiosome (peribacteroid) membrane, the glyceollin concentration range increased by a factor of about 100, similar to a range in-

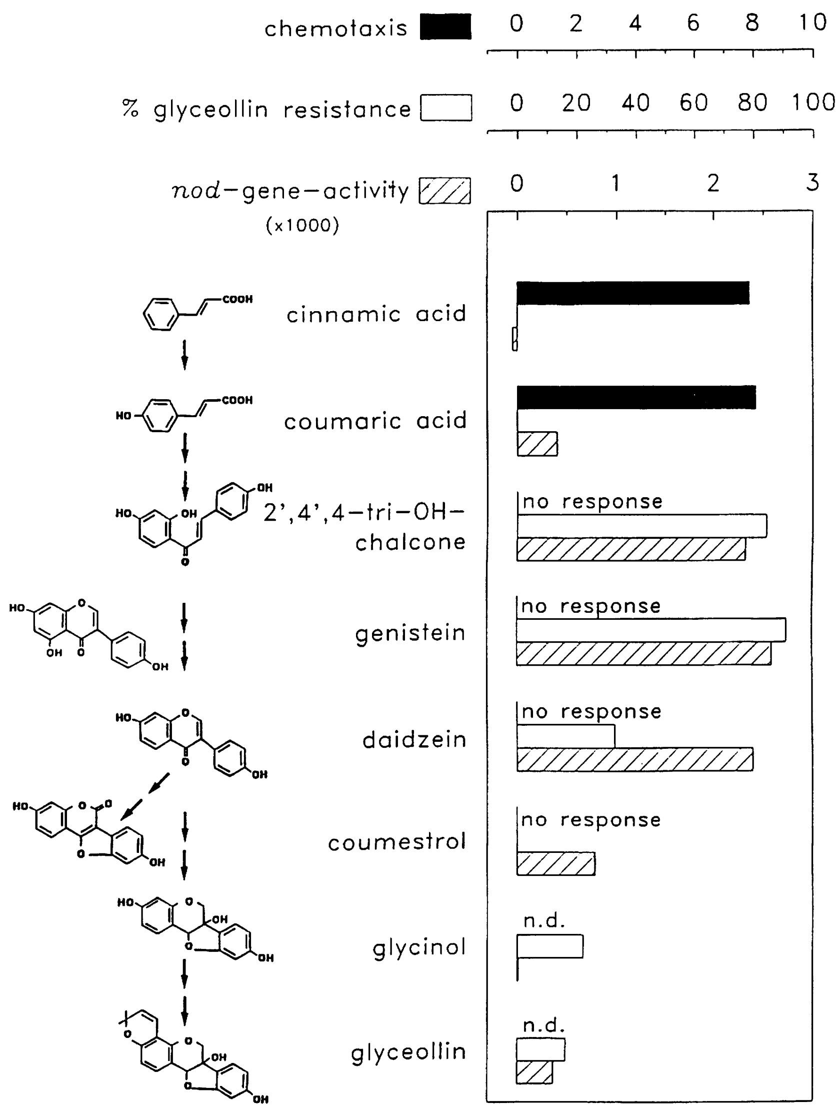

Fig. 9: Differential inductions of symbiotic activities in *Bradyrhizobium japonicum* by intermediates of the pterocarpan and the coumestan biosynthetic pathways. Test compounds were applied at a concentration of 10 μM. *Nod* gene activity is expressed in β-galactosidase units (in thousands) according to Miller (1972). Glyceollin resistance is expressed as the percentage of cells that survived a 3-h incubation in 300 μM glyceollin. The chemotaxis data are derived from Kape et al. (1991 and 1992) and are expressed as the chemotactic ratio at the concentration that elicited the chemotactic peak response. n.d., not determined (modified from Parniske, 1991).

duced by phytopathogenic fungi (Werner et al., 1985; Parniske et al., 1991 a).

The symbiotic communication by phenylpropane metabolites

The thus far established results on chemotaxis, *nod* gene inducing activity and phytoalexin inducing activity of metabolites of the phenylpropane pathway in the *Rhizobium/Bradyrhizobium* – legume interaction are summarized in Fig. 9 (references are given in the figure legend). The results shown in Fig. 2 indicate that for the different root zones the concentrations of these compounds vary to a large extent. A similar scheme for the VA-mycorrhiza interaction is not available up to now, since only some effects of phenylpropane metabolites on spore germination and hyphal growth are known. Results are not yet available on compounds that influence the attraction of infecting hyphae to the rhizodermis cells. Also, there is no sufficient data on defence reactions of the host during the first stages of infection and effects of flavonoids on fungal differentiation into arbuscules, vesicles, and spores.

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