Defence Reactions in *Rhizobium*-Legume Symbiosis: Phytoalexin Concentration in *Vicia faba* Nodules is Affected by the Host Plant Genotype

Andreas B. Wolff and Dietrich Werner

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

Z. Naturforsch. **45c**, 958–962 (1990); received August 4, 1989/June 21, 1990

Vicia faba, Rhizobium leguminosarum, Nodules, Phytoalexin

In the *Vicia faba-Rhizobium leguminosarum* symbiosis defence reactions are established by a variable phytoalexin concentration in the nodules. No differences were observed between effective (fix$^+$) and ineffective (fix$^-$) nodules. In nodules of field grown plants the average phytoalexin concentration was about 3 times higher than that from sterile pot-cultured plants infected with different *Rhizobium leguminosarum* strains. A great variability of phytoalexin accumulation in nodules of single plants was observed. In field grown plants about 60% of the nodules of each plant had very low levels (<50 µg wyerone · g nodule fresh weight^{-1}) the other 40% had levels between 100 and 600 µg wyerone · g nodule fresh weight^{-1} indicating different defence reactions in the nodules distributed over the root system of one plant. Phytoalexin accumulation was more affected by the cultivar of the host plant than by the infecting *Rhizobium* strain. Two inbred lines had the lowest concentration in nodules.

Introduction

Phytoalexins are antimicrobial compounds with a low molecular weight. They are synthesized and accumulated in plants exposed to microorganisms or other elicitors such as heavy metal salts, detergents or wounding substances. Precursors of isoflavonoid phytoalexins have found a new essential role in communication of symbiotic bacteria in the genera *Rhizobium, Bradyrhizobium* and *Sinorhizobium* with their respective hosts [1–4]. In contrast to other legumes which mainly synthesize isoflavonoids as phytoalexins, *Vicia faba* produces furanoacetylenes. The main phytoalexins in faba beans are wyeronic acid, dihydrowyeronic acid, wyerone, dihydrowyerone, wyerol, dihydrowyerol, wyerone epoxide and wyerol epoxide. In leaves and pods of *Vicia faba* wyeronic acid and dihydrowyeronic acid are the major components, whereas in seeds mainly wyerone and dihydrowyerone are accumulated [5]. In nodules wyerone is the dominant phytoalexin. The induction of phytoalexin production is independent of the type of phytoalexins synthesized but follows similar general rules and stimuli [6, 7]. It is only part of the sequence of several defence reactions [8]. In phytopathogenic but incompatible (plant resistant) interactions the accumulation of phytoalexins is limited in a small area around the infection site [9, 10]. Tissue-specific mechanisms of phytoalexin induction and synthesis have also successfully been studied with tissue cultures [11, 12]. Very specific elicitors such as the peptide toxin victorin from *Helminthosporium victoriae* have been characterized [13]. The accumulation of the phytoalexin produced in this system (avenalumin) strictly depends on a very narrow range of concentration of this elicitor.

The accumulation of phytoalexins in tissues of *Vicia faba* can be used as a biochemical marker for different characters of cultivars or inbred lines. A correlation was found between phytoalexin accumulation and resistance of *Vicia faba* cultivars to phytopathogenic fungi [14]. The accumulation of phytoalexins in *Vicia faba* in tissue cultures can also be used as a marker for the regeneration capacity of these cultures [15].

Phytoalexins may be also a marker for the compatibility of a symbiosis. Werner *et al.* [16] found a high level of phytoalexins in nodules of *Glycine max cv.* Mandarin infected with the ineffective strain (fix$^-$) of *Bradyrhizobium japonicum* 61-A-24, characterized by an early degradation of the peribacteroid membrane separating the microsymbiont from the host cytoplasm. The concentration of phytoalexins in soybean nodules with a stable peribacteroid membrane, *i.e.* in compatible symbiosis, is very low.

Reprint requests to Dr. A. B. Wolff.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0900–0958 $ 01.30/0

In this study we can demonstrate that the symbiotic bacterium *Rhizobium leguminosarum* elicites a defence reaction in *Vicia faba* but that the phytoalexin concentration in nodules is low. A significant variability of phytoalexin accumulation in nodules of different cultivars and in single nodules from field-grown plant is demonstrated, indicating, so far unknown, differences within the nodule population of a single plant in the field.

Materials and Methods

Plant material, Rhizobium strains and inoculation of faba bean

Seeds of faba bean (*Vicia faba* L.) were received from Norddeutsche Pflanzenzucht, 2331 Holtsee (F.R.G.). Strains of *Rhizobium leguminosarum* PF 2, B 10, and F 28 were obtained from Dr. Lotz, University of Erlangen, F.R.G., and strain PRE from Dr. Lie, Agricultural University, Wageningen, Netherlands.

Vermiculite was sterilized for 2 h at 200 °C and inoculated with strains of *Rhizobium leguminosarum* PRE, B 10, PF 2 and F 28 grown in 20 E medium [17]. The seeds of *Vicia faba cv.* Kristall and *cv.* TP 667 were planted in the field and inoculated with the mixture of vermiculite and *R. leguminosarum* strains. The titre was about 10^{10} bacteria per seed.

Cultivation and inoculation of faba bean in pot-cultures was done as described by Werner *et al.* [17]. The growth conditions in the phytotron were 16 h light, 18 °C, 70% rel. moisture and 8 h dark, 15 °C, 70% rel. moisture. Phytotron and field-grown plants were harvested after 7 weeks.

Extraction, identification and quantification of phytoalexins

Extraction of tissues was as described by Thynn *et al.* [18], identification and quantification of phytoalexins by HPLC according to Wolff *et al.* [15]. For determination of the phytoalexin content of single nodules these were homogenized in 1.5 ml Eppendorf cups with 300 – 500 µl ethanol, incubated over night and centrifuged 2 min at 10,000 × *g*. The supernatant was used directly for HPLC analysis of phytoalexins.

Light microscopical identification of fungal infections

Roots and nodules were examined for fungal infection by light microscopy as described by Brundrett *et al.* [19].

Results

Wyerone content in nodules of field-grown plants of *Vicia faba* and pot-cultures differed considerably. In addition to the growth conditions, phytoalexin synthesis was also affected by the *Vicia faba* cultivar.

The average concentration in 7 week-old nodules of field-grown plants was about 80 µg wyerone$\cdot \mathrm{g}^{-1}$ nodule fresh weight for *cv.* Kristall and 50 µg for *cv.* TP 667 (Fig. 1) and <35 µg for pot-cultures infected with *Rhizobium leguminosarum* strains. In nodules from other cultivars even higher figures (>150 µg wyerone$\cdot \mathrm{g}^{-1}$ nodule fresh weight) were found (Fig. 3). As shown in Fig. 1, plants inoculated with defined *Rhizobium leguminosarum* strains in the field had a lower level of wyerone than the not inoculated control plants, infected with endogenous *Rhizobium* strains from

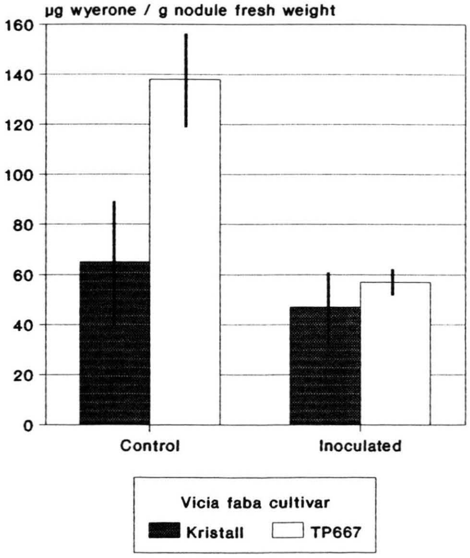

Fig. 1. Phytoalexin (wyerone) in nodules from field-grown plants of *Vicia faba* inoculated with *Rhizobium leguminosarum* and non-inoculated control plants of *cv.* Kristall and TP 667.

the soil. The cultivar effect on phytoalexin accumulation could is demonstrated in Fig. 1 and Fig. 3.

The concentration of wyerone in different nodules of one plant is not uniform. When single nodules of one plant were analyzed, a great variability in phytoalexin content was found. This effect was more obvious in field-grown plants than in pot-cultures. While about 60% of the nodules of field-grown plants had levels smaller than 50 μg wyerone$\cdot$g^{-1} nodule fresh weight, 35% had up to 300 μg and about 5% had about 600 μg (Fig. 2). In nodules from pot-cultured faba beans infected with defined *Rhizobium leguminosarum* strains in no case more than 100 μg wyerone$\cdot$g^{-1} nodule fresh weight was found. The wyerone content of most nodules (90%) was lower than 50 μg wyerone$\cdot$g^{-1} nodule fresh weight (Fig. 2).

In most cultivars the phytoalexin concentration was higher in roots than in nodules (Fig. 3). This is remarkable, since 1 g of legume nodules includes more than 10^{11} bacteria inside the tissue, in the compartments of the now so-called symbiosome [20]. Exceptions were found in the cultivars "Alfred" and "Kompakta" with significant higher wyerone concentration in nodules compared to roots. This may be related to the observation that the peribacteroid membranes of the symbiosome opens in the vicinity of the host cell nucleus in some *Vicia faba* nodules [21]. The inbred lines TP215 (No. 3 in Fig. 3) and 121 (No. 6 in Fig. 3) had very low wyerone concentrations in nodules as well as in roots. The effect of different *Rhizobium leguminosarum* strains on phytoalexin accumulation in nodules is very low, with variations of not more than 20 μg wyerone per g nodule fresh weight (Fig. 4).

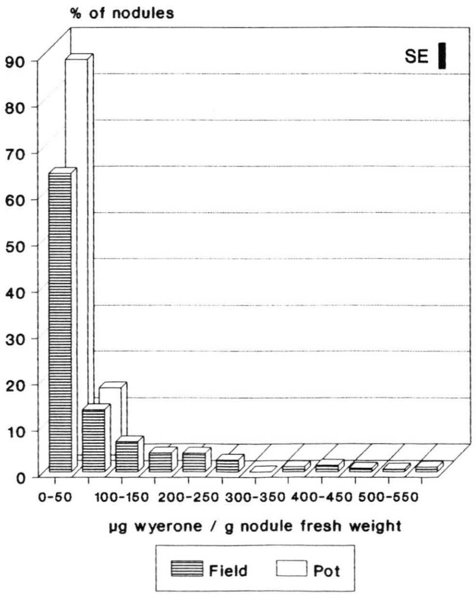

Fig. 2. Phytoalexin (wyerone) in nodules from field-grown plants of *Vicia faba cv.* Kristall and *cv.* TP667. Pot-cultured plants were infected with *Rhizobium leguminosarum* strain F28 and strain PRE.

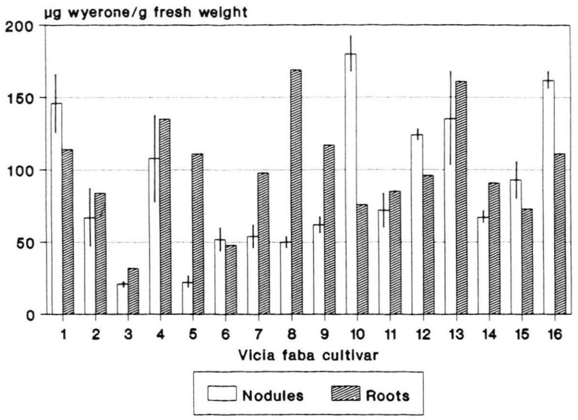

Fig. 3. Phytoalexin (wyerone) accumulation in roots and nodules of different cultivars or inbred lines from field-grown faba beans. *Vicia faba* cultivars or inbred lines: 1: HV130, 2: TP15700, 3: TP215, 4: STP470, 5: 120, 6: 121, 7: ST693/84, 8: Minica "115", 9: ST8247, 10: Alfred, 11: LG4/17/8, 12: Troy, 13: Kristall, 14: Optica, 15: White Flower, 16: Kompakta. (Plant material was kindly received from Dr. Ebmeyer, Universität Göttingen, F.R.G.).

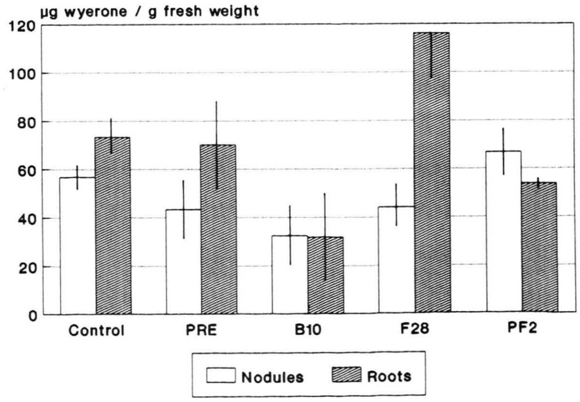

Fig. 4. Phytoalexin (wyerone accumulation in roots and nodules from *Vicia faba cv.* TP667 inoculated with different *Rhizobium leguminosarum* strains (PRE, B10, F28, PF2).

A concentration of phytoalexins comparable to a typical attack by phytopathogenic fungi has only been observed in nodules with a completely unstable peribacteroid membrane of the symbiosome [16]. The same result can be observed in a combination of fix$^+$ strains of *Bradyrhizobium japonicum* with *Glycine soja*, but not with *Glycine max* [22]. As in soybeans [16], we found no correlation between nitrogen fixing (fix$^+$) strains or fix$^-$ strains and phytoalexin accumulation in the symbiotic organs of faba beans.

Roots and nodules were checked for fungal infection. No infection in the nodules could be observed whereas some roots were infected.

Discussion

Little is know about defence reactions in the development of the symbiosis between Fabales and rhizobia. More than 90% of the primary infections of root hairs are abortive [23] indicating a strong defence response by the plant. This was confirmed by electron microscopy studies by Djordjevic *et al.* [24], observing a hypersensitive reaction in root hairs after infection by rhizobia.

Our results indicate that also in legume nodules a slight defence reactions occurs together with an accumulation of phytoalexins. This very low level of phytoalexins was observed in sterile grown plants infected by defined *Rhizobium leguminosarum* strains. In 90% of the nodules of one plant $<50\ \mu g$ wyerone$\cdot g^{-1}$ nodule fresh weight was found. Mansfield *et al.* [25] reported that in cotyledons infected by *Botrytis cinerea* phytoalexin levels can increase to about 1300 μg wyerone$\cdot g^{-1}$ fresh weight. In *Vicia faba* meristem cultures infected by *Botrytis cinerea* up to 450 μg wyerone$\cdot g^{-1}$ fresh weight were determined [14]. The phytoalexin concentration in about 30% of the nodules was higher under field conditions than in pot-cultured plants grown under sterile conditions in the phytotron. It is unknown, whether this is due to elicitors from partly incompatible *Rhizobium* strains infecting the plants from soil or due to other stress factors.

Phytoalexins such as phaseollin produced in bean hypocotyls can be metabolized *e.g.* by *Fusarium solani* f. sp. *phaseoli* [26, 27]. Detoxification of phytoalexins is a common mechanism of plant pathogens to break the defence reaction of the host plant. Wyerone has, compared to isoflavonoid phytoalexins, a rather limited stability. An additional detoxifying effect by the symbiotic nodule bacteria is an unproven possibility.

We observed that nodules were not infected by fungi (*e.g.* mycorrhiza) although some roots were invaded. These results are consistent with observations of Bethlenfalvey *et al.* [28] that nodules of soybeans are better protected against fungal infections than roots. In contrast to the roots, nodules have an outer cork layer which perhaps prevents the invasion of fungi. The results confirm the idea that in a large number of cultivar/strain combinations, the massive invasion of the host tissue by symbiotic microorganisms does not elicit a strong defence reaction in the compatible interaction with stable structures (symbiosomes). However, under field conditions a significant percentage of nodules produces such a defence reaction, indicating differences between laboratory cultures and soil systems.

Acknowledgements

We thank the BMFT for the project "Wechselwirkungen Pflanzen – Mikroorganismen" (Project No. 03 189 66 B).

[1] S. R. Long and J. Cooper, in: Molecular Genetics of Plant-Microbe Interactions (R. Palacios and D. P. S. Verma, eds.), APS Press, St. Paul 1988.

[2] H. Grisebach, L. Edelmann, D. Fischer, G. Kochs, and R. Welle, in: Signal Molecules in Plants and Plant-Microbe Interactions (B. J. J. Lugtenberg, ed.), NATO ASI Series H, **Vol. 36,** Springer Verlag, Berlin, Heidelberg, New York 1989.

[3] E. Appelbaum, in: Molecular Biology of Symbiotic Nitrogen Fixation (P. M. Gresshoff, ed.), CRC Press, Boca Raton 1990.

[4] A. Economou, W. D. O. Hamilton, A. W. B. Johnston, and J. A. Downie, EMBO J. **9,** 349 (1990).

[5] J. L. Ingham, in: Phytoalexins (J. A. Bailey and J. W. Mansfield, eds.), Blackie and Son Ltd., Glasgow, London 1982.

[6] A. G. Darvill and P. Albersheim, Ann. Rev. Plant Physiol. **35,** 243 (1984).

[7] J. Ebel, Ann. Rev. Phytopathol. **24,** 235 (1986).

[8] K. Hahlbrock, N. Arabatzis, M. Becker-Andre, H.-J. Joos, E. Kombrink, M. Schröder, G. Strittmatter, and J. Taylor, in: Signal Molecules in Plants and Plant-Microbe Interactions (B. J. J. Lugtenberg, ed.), NATO ASI Series H, **Vol. 36,** Springer Verlag, Berlin, Heidelberg, Stuttgart 1989.

[9] J. W. Mansfield, J. A. Hargreaves, and F. C. Boyles, Nature **252,** 316 (1974).

[10] M. G. Hahn, A. Bonhoff, and H. Grisebach, Plant Physiol. **77,** 591 (1985).

[11] B. Vornam, H. Schön, and H. Kindl, Plant Mol. Biol. **10,** 235 (1988).

[12] J. Ebel, E. G. Cosio, M. Feger, D. Grab, and H. Habereder, in: Signal Molecules in Plants and Plant-Microbe Interactions (B. J. J. Lugtenberg, ed.), NATO ASI Series H, **Vol. 36,** Springer Verlag, Berlin, Heidelberg, Stuttgart 1989.

[13] S. Mayama, T. Tani, T. Ueno, S. L. Midland, J. J. Sims, and N. T. Keen, Physiol. Mol. Plant Pathol. **29,** 1 (1986).

[14] M. Thynn, A. B. Wolff, E. Görge, and D. Werner, Z. Naturforsch. **44c,** 237 (1989).

[15] A. B. Wolff, M. Thynn, E. Görge, and D. Werner, Z. Naturforsch. **43c,** 71 (1988).

[16] D. Werner, R. B. Mellor, M. Hahn, and D. Grisebach, Z. Naturforsch. **40c,** 179 (1985).

[17] D. Werner, J. Wilcockson, and E. Zimmermann, Arch. Microbiol. **105,** 27 (1975).

[18] M. Thynn, A. Wolff, E. Görge, and D. Werner, Z. Naturforsch. **44c,** 237 (1989).

[19] M. C. Brundrett, Y. Piche, and R. L. Peterson, Can. J. Bot. **62,** 2128 (1983).

[20] L. Evans Roth and G. Stacey, Cell Biol. **49,** 13 (1989).

[21] A. Wolff, R. Mörschel, C. Zimmermann, M. Parniske, S. Bassarab, R. B. Mellor, and D. Werner, in: Physiological Limitations and the Genetic Improvement of Symbiotic Nitrogen Fixation (F. O'Gara *et al.,* eds.), Kluwer Academic Publ., Dordrecht 1988.

[22] M. Parniske, C. Zimmermann, P. B. Cregan, and D. Werner, Botanica Acta **103,** 143 (1990).

[23] D. Werner, Die pflanzlichen Symbiosen, Georg Thieme Verlag, Stuttgart, New York 1987.

[24] S. P. Djordjevic, R. W. Ridge, H. Chen, J. W. Redmond, M. Batley, and B. G. Rolfe, J. Bacteriol. **170,** 1848 (1988).

[25] J. W. Mansfield, A. E. A. Porter, and R. V. Smallman, Phytochemistry **19,** 1057 (1980).

[26] J. Van Den Heuvel and H. D. Van Etten, Physiol. Plant Pathol. **3,** 327 (1973).

[27] H. D. Van Etten, D. E. Matthews, and P. S. Matthews, Ann. Rev. Phytopathol. **27,** 143 (1989).

[28] G. J. Bethlenfalvay, M. S. Brown, and A. E. Stafford, Plant Physiol. **79,** 1054 (1985).