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Plant Defense Responses of Host Plants with Determinate Nodules Induced by EPS-Defective exoB Mutants of Bradyrhizobium japonicum

Martin Parniske, Petra E. Schmidt, Kerstin Kosch, and Peter Müller

Fachbereich Biologie der Philipps-Universität, Karl-von-Frisch-Straße, D-35043 Marburg/Lahn, Germany

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The symbiotic phenotype of exoB mutants ΔP5 and ΔP22 of Bradyrhizobium japonicum 110spc4 was analyzed on the host plants Glycine max and G. soja. The extent of the symbiotic defects was host dependent. In combination with G. max, the B. japonicum exoB mutants induced the formation of effective nodules. Infection threads were found in the central nodule tissue of developing nodules, similar to wild-type infected nodules. However, in early stages of the interaction between the mutants and G. max, plant defense reactions occurred, among which phytoalexin accumulation was the earliest effect observed. Later the rhizodermis was disrupted by longitudinal cracks caused by cortical cell proliferations, and rhizodermal strips were frequently peeled off the growing nodules. Our results indicate that the intact EPS of B. japonicum is necessary for the prevention of plant defense reactions during early interaction with soybean. Combinations between G. max and B. japonicum exoB mutants seemed to be impaired only transiently, since they resulted in effective nodule formation. However, enhanced concentrations of chitinase within the central nodule tissue of B. japonicum exoB mutant induced G. max nodules proved the occurrence of plant defense reactions also in later steps of nodule development. On G. soja, B. japonicum exoB mutants lost their infectivity and induced the formation of white, uninfected and ineffective nodulike structures at the base of lateral roots.

Additional keyword: glyceollin.

Nitrogen-fixing root nodules are the result of a complex molecular communication process during the symbiotic interaction of rhizobia and legumes. Depending on the host plant species, various types of nodules are formed that can be subdivided into two principal groups. Determinate nodules are spherical, and the central meristematic activity of plant nodule cells stops at a certain predetermined developmental stage. In contrast, indeterminate nodules are cylindrical and have a persistent apical meristem. Apart from the ability to induce meristematic activity in the host root, rhizobia have evolved mechanisms to infect plant cells. The work of several laboratories has unambiguously shown that specific rhizobial exopolysaccharide (EPS) structures are essential prerequisites for a successful infection of indeterminate nodule type legumes, e.g., Leucaena, Medicago, Pismum, Trifolium, or Vicia species by rhizobia (Borthakur et al. 1986; Chakravorty et al. 1982; Diebold and Noel 1989; Hotter and Scott 1991; Leigh et al. 1987; Müller et al. 1988; for a recent review see Leigh and Coplin 1992). EPS mutants of the corresponding microsymbionts R. loti, R. meliloti, or R. leguminosarum induce the formation of uninfected nodulike structures that are devoid of bacteroids. Increasing evidence suggests that EPS are involved in the prevention of plant defense responses (Ahborn and Werner 1991; Pühler et al. 1991; Niehaus et al. 1993).

In determinate nodule-type legumes such as soybean, the role of EPS is less clear. Rhizobial strains, which could effectively nodulate both determinate and indeterminate types of host plants, were of particular interest to address this question. For instance, EPS- mutants of the broad host range Rhizobium sp. NGR234 formed defective nodules on the indeterminate nodulating host Leucaena but formed normal, effective nodules on several tropical legumes of the determinate nodulating type (Chen et al. 1985). Hotter and Scott (1991) reported on Exo- mutants of Rhizobium loti which formed defective nodules on Leucaena but normal nodules on Lotus. The conclusion drawn from these analyses was that EPS is not essential for the formation of effective nodules on determinate nodulating legumes. However, as previously shown, at least in symbiosis with the determinate nodulating legume soybean, rhizobial EPS is not without function. By the construction of specific and genetically defined EPS mutants of B. japonicum, it was demonstrated that the elimination of the exoB gene of B. japonicum leads to specific mutants that form an altered EPS, whereas LPS remained intact. These mutants nodulated soybeans with a delay and a concomitant dramatic loss of competition (Parniske et al. 1993). This symbiotic phenotype pointed to a significant function of EPS in the early stages of the symbiotic interaction. Here a more detailed analysis of the symbiotic phenotype of B. japonicum exoB mutants raised the question for the specific function of EPS in the infection process of determinate nodules.

The severity of the aberrant phenotypes depended on the host plant. On Glycine soja, B. japonicum exoB mutants induced meristematic activity but nodule infection was blocked. On G. max, several plant defense reactions were observed. In spite of these, the mutants succeeded in reaching the central...
Figs. 1–6. 1, Effective nodule of *Glycine soja* PI468397 induced by *Bradyrhizobium japonicum* 110spc4, 20 dpi. × 10, bar = 1 mm. 2, Nodule-like structures of *G. soja* PI468397 induced by *B. japonicum* ΔP22, 20 dpi. × 5, bar = 2 mm. 3, Nodule of *G. max* 'Preston' induced by *B. japonicum* ΔPS, 20 dpi. Lateral view. Typically, the outer cell layers separate from the underlying cortex tissue very early in development and are peeled off as the nodule expands (indicated by a bidirectional arrow). ×10, bar = 1 mm. 4, Nodules of *G. max* 'Preston' induced by *B. japonicum* ΔPS, 20 dpi. View from the top. Longitudinal cracks in the rhizodermis frequently occur (bidirectional arrows). ×7.5, bar = 1.5 mm. 5, Micrograph of a longitudinal section through a *G. max* 'Preston' nodule induced by *B. japonicum* 110spc4, 15 dpi. Note that the meristem (m) is shielded from direct contact with the environment by non-meristematic, continuous layers of cortical cells. Bar = 50 μm. 6, Micrograph of a longitudinal section through a *G. max* 'Preston' nodule primordium induced by *B. japonicum* ΔPS, 15 dpi. The lack of the cell layers at the tip of the primordium, normally enclosing the central meristem in a wild type induced nodule (Fig. 5), is apparent. Bar = 50 μm.
nodule tissue via infection threads to establish an effective symbiosis. Nodule formation, however, occurred somewhat delayed. The observations are summarized in a working hypothesis for the biological role of *B. japonicum* EPS in the infection process with determinate type host plants.

**RESULTS**

Morphological analysis of soybean nodules induced by *exoB* mutants reveals symptoms of plant defense reactions.

The observation that *B. japonicum* *exoB* mutants nodulated soybean with a delay of approximately 5 days with respect to the wild type, indicated a disturbance in the early stages of the symbiotic interaction (Parniske *et al.* 1993). The symbiotic phenotype of these mutants was analyzed in more detail to elucidate the characteristics of the mutant phenotype. Since the intensity of plant defense reactions has been shown to depend on the plant species (Parniske *et al.* 1990), two different plant species, *Glycine max* and *G. soja*, were tested for their interaction with *B. japonicum* *exoB* mutants. In Figures 1–4, wild-type and mutant-induced nodules 20 dpi on *G. soja* (Figs. 1 and 2) and mutant induced nodules on *G. max* (Figs. 3 and 4) are shown. On *G. max*, younger wild-type nodules were surrounded by an intact epidermis which was in continuum with the rhizodermis. This outer cell layer was further differentiated in later developmental stages, resulting in the formation of lenticels at the periphery of the emerging nodules. Longitudinal cracks in the rhizodermis appeared only in the vicinity of old nodules and were similar to the local cracks around the sites of emerging lateral roots.

The morphology of nodules induced by *B. japonicum* *exoB* mutants ΔP5 or ΔP22 on soybean (*G. max*) appeared to be identical and could be macroscopically distinguished from wild-type nodules. Mutant nodules exhibited unusual but characteristic features, differing greatly from one nodule to the next. As shown in Figures 3 and 4, the rhizodermis exhibited longitudinal cracks around the emerging *G. max* nodules induced by the mutant strains. The rhizodermis and adjacent cell layers were ruptured and stripped off due to the expansion of the growing nodule primordium. Occasionally, the cortical cells of the developing nodule were visible as undifferentiated, loosely associated outgrowths of the root, which were apparently uninfected at that stage (Fig. 4). As a consequence of the loss of the outer cell layers, and in contrast to wild-type nodules (Fig. 5), the cortex of mutant nodules was directly exposed to the environment (Fig. 6). Therefore, the nodule cortical cells came in direct contact with the bacterial inoculum (Fig. 7). Remnants of collapsed cells were occasionally found at the outermost distal parts of the cortical tissue (Figs 8 and 9). The outermost cell layer of this tissue had thickened cell walls. Obviously these cells functionally substituted for the regular rhizodermis. Locally restricted cell death of rhizodermal cells and cell wall thickenings of cortical cells were possibly symptoms of structural plant defense responses involving only the outermost cell layers.

**Phytoalexin accumulation in soybean root exudate induced by *B. japonicum* *exoB* mutants.**

Since the morphological analysis of soybean nodules induced by *B. japonicum* *exoB* mutants revealed symptoms of plant defense responses, we analyzed this interaction for the occurrence of other features usually associated with plant defense response. For example, when soybean roots are challenged with phytopathogenic organisms such as *Phytophthora megasperma*, the production of phytoalexins, antimicrobial low molecular weight compounds, is induced (Schmidt *et al.* 1992). The major phytoalexin in soybean is the isoflavonoid glyceollin. As reported earlier (Schmidt *et al.* 1992)
glyceollin accumulation reaches its maximal value as early as 10 hr after inoculation with *B. japonicum* wild-type strain 110spc4, but the concentration was found to be at a considerably lower level compared to elicitation by *P. megasperma*.

The concentration of glyceollin was analyzed during the interaction of soybean with EPS mutants of *B. japonicum*. When soybean (*G. max*) seedlings were axenically incubated in MES buffer, only negligible amounts of glyceollin were detectable in root exudate. In the presence of the *B. japonicum* exoB mutants ΔP5 or ΔP22, enhanced glyceollin concentrations were found in the soybean root exudate as early as after 18 hr of coincubation. Phytoalexin accumulation increased with longer incubation times. After 72 hr, a concentration of glyceollin about ten times higher was measured in the root exudate compared to incubation with the parent strain *B. japonicum* 110spc4 (Fig. 10). The observation that glyceollin production increased within the first day after inoculation with *B. japonicum* exoB mutants shows that an early plant defense reaction has occurred.

**Immunolocalization of chitinase in the infected zone of soybean nodules induced by *B. japonicum* exoB mutant ΔP22.**

Nodules of *G. max* at 21 dpi with *B. japonicum* 110spc4 or *B. japonicum* exoB mutant ΔP22 were analyzed for the presence of further symptoms of plant defense reactions in more advanced steps of nodulation. Tissue prints of nodules on nitrocellulose membrane were probed against a bean chitinase antiserum. In wild-type infected nodules, cross-reactive material against bean chitinase antibodies was detectable in the cortex (Fig. 11A). In contrast, in nodules resulting from infection with *B. japonicum* exoB mutant ΔP22 cross-reactive material against bean chitinase was additionally found in the infected zone (Fig. 11B). The reaction was found to be locally restricted. Some of the wild-type nodules exhibited a similar pattern, although less frequently and at lower intensities (data not shown). A quantitation of the effect showed that the reaction occurred in 100% of mutant nodules (46 prints tested) and only in 17% (69 prints tested) of wild-type infected nodules. This was a strong indication that the nodulation defects of the *B. japonicum* exoB mutant not only affected the early steps of infection but that defense-related reactions occurred also in some later stages of nodule development.

**Blocked infection of Glycine soja PI468397 by exoB mutants.**

It is known that the induction of plant defense responses in already developed nodules depends on the specific combination of an inoculant strain and the host plant genotype (Parniske et al. 1998). Glycine soja PI468397, inoculated with *B. japonicum* 110spc4 and grown in petri dishes, had formed four to five effective nodules per plant at 20 dpi. An effective nodule is shown in Figure 1. At the same time, several non-infected nodulelike structures were observed next to the effective nodules on the same root systems. The observation of small, white, ineffective nodules indicated that the combination between *G. soja* and *B. japonicum* strain 110spc4 was not an optimal one. On this suboptimal host plant, *G. soja* PI468397, the aberrant nature of the symbiotic phenotype of *B. japonicum* exoB mutants was even more pronounced, compared to the optimal interaction with *G. max*. Numerous, small, white nodulelike structures were observed which frequently were located at the base of lateral roots. Figure 2 shows a part of the root with several lateral roots and a number of such nodulelike structures.

No nitrogenase activity, as indicated by the acetylene reduction assay, was detectable in *G. soja* plants inoculated with *B. japonicum* exoB mutants. Following surface sterilization, no rhizobia could be reisolated from the small white nodules, indicating that they were not colonized by the mutant strains. As found with *G. max* exclusively in combination with *B. japonicum* exoB mutants, the expanding root nodule primordia of *G. soja*, following inoculation with *B. japonicum* wild-type or exoB mutant, lead to longitudinal rupturing of the rhizodermis (Fig. 2). In certain instances, a prolonged incubation time (4–5 wk) in Leonard jars, when plants were already suffering severely from nitrogen deficiency, as indicated by yellow leaves and poor growth, effective nodules occasionally developed on *G. soja* plants infected with *B. japonicum* exoB mutants. Reisolation of the bacteria from these nodules confirmed that all colonies arising on agar plates without antibiotics (kanamycin) were also resistant against kanamycin as expected for *B. japonicum* exoB mutants, and no kanamycin-sensitive colonies (wild-type strain) were obtained. These observations indicated that the nodulelike structures normally observed in this combination, occasionally were infected in more advanced developmental stages, but these events have to be regarded as the rare exception rather than the rule.

**DISCUSSION**

The construction of *B. japonicum* exoB mutants that produce a structurally modified, galactose-free EPS but unaltered LPS has previously been reported (Parniske et al. 1993). The delayed nodulation phenotype on soybean (*G. max*) and the reduced competitiveness of these *B. japonicum* exoB mutants...
indicated that a correct EPS structure is essential during the early stages of the symbiotic interaction. The present study attempted to define more precisely the effects of a defective EPS produced by genetically defined _B. japonicum exoB_ mutants on the development of determinate nodules. Morphological analyses of soybean nodules induced by _B. japonicum exoB_ mutants revealed several characteristic features. Single collapsed plant cells in the outermost cell layers of soybean roots and nodule primordia were found to be typical for this interaction. Massive thickenings of root cell walls in direct contact with the inoculant mutant strains frequently occurred. Furthermore, about 10 times higher concentrations of the phytoalexin glyceollin were detected in seedlings inoculated with _B. japonicum exoB_ mutants, compared to the wild type.

The defense response of _G. max_ nodules infected with _B. japonicum exoB_ mutant ΔP22 gives another proof for plant defense reactions in advanced steps of nodule development (21 dpi). Obviously, the altered composition of bradyrhizobial carbohydrate surface compounds is detected by the plant, in the infected and differentiated plant tissue. Therefore, a role of bacterial EPS also in later stages of the development of determinate soybean nodules has to be postulated. Plant chitinases often are associated with plant defense reactions (Boller 1988). Furthermore, chitinases have been reported to inhibit the growth of certain fungi (Schlumbera et al. 1986) and often have lysozyme activity as well, indicating that they may function in defense reactions directed against bacteria (Boller 1988). Enhanced chitinase levels in transgenic plants have been demonstrated to reduce the damage caused by pathogens (Broglie et al. 1991). In another experimental approach by Sirit et al. (1993), the expression of _Serratia marcescens_ chitinase gene in _Rhizobium meliloti_ during symbiosis on alfalfa roots suggested an increased plant resistance to pathogens. The bacterial type of chitinases is related in its sequence to class V of tobacco chitinases which has been cloned and sequenced by Melchers et al. (1994). In the infected zone of the _Glycine max_ 'Preston' nodules, chitinase was also found in the central nodule tissue of ineffective nodules colonized by bacteria unable to fix nitrogen symbiotically (Staehelein et al. 1992).

In a recent review about plant chitinases, Collinge et al. (1993) have outlined that chitinase expression is also under developmental control in certain organs and tissues and seems to have an important function in additional non-defensive roles. Of particular interest is the observation that chitinase can inactivate the chitinlike nodulation factors produced by _Rhizobium_ and _Bradyrhizobium_ strains (Roche et al. 1991). Cytochemical and immunological characterization of abortive infections during the _Rhizobium meliloti_-alfalfa symbiotic interaction have lead to the idea that acidic chitinases which accumulate in cells with a hypersensitive reaction, could spe-
specifically hydrolyze Nod factors (Vasse et al. 1993). The finding that structural modifications in Rhizobium meliloti nodulation factors influence their stability against root chinatases have supported the idea that the action of chinatases may determine the activity of rhizobial nodulation factors (Staehelin et al. 1994).

When B. japonicum exoB mutants were inoculated onto G. soja seedlings, small white nodules were formed which were not colonized by the bacteria. This shows that the severity of the phenotype depended on the host plant species. The uninfected nodules of G. soja, induced by B. japonicum exoB mutants had some features in common with the aberrant nodules of G. max. In both cases the outer cell layers were found to separate from the underlying cortex tissue, suggesting the induction of similar plant defense responses in both plant species. On G. soja, the symbiotic interaction of the B. japonicum exoB mutants appeared to be disturbed most pronouncedly, since the mutants did not invade the central nodule tissue. However, one has to take into account that G. soja PI468397 is only poorly nodulated by B. japonicum strains (Parniske et al. 1990). This particular plant genotype appears to be better adapted to fast-growing soybean rhizobia, R. fredii (Keyser and Cregan 1984).

As previously shown, plant defense responses may also occur in more advanced stages of soybean nodule development. This phenomenon was found in very restricted, specific combinations of host cultivar and B. japonicum strain. For example, specific strains of B. japonicum induced a hypersensitive response in the nodules of G. soja PI468397, but not in nodules of G. max (Parniske et al. 1990). A similar observation was made in G. max nodules infected with a nifA-mutant of B. japonicum (Parniske et al. 1991b) or specific isolates of B. japonicum (Werner et al. 1985). Depending on the host/strain combination, plant defense responses to rhizobial infection are observed in very early or in later stages of nodule development. Furthermore, in well-adapted combinations of host plant and invading microsymbionts, e.g., G. max/B. japonicum or M. sativum/R. meliloti, basic plant defense reactions like glyceollin (Schmidt et al. 1992) or medicarpin accumulation (Dakora et al. 1993) were observed at low levels. These processes seem to be regulated by fine-tuning mechanisms as recently shown by Vasse et al. (1993). They demonstrated that abortive infections of R. meliloti on alfalfa were accompanied by the accumulation of phenolic compounds and defense-related proteins in single affected cortex cells. (Brady)rhizobial surface carbohydrates like EPS or LPS appear to be involved in the well-balanced communication processes between the symbiotic partners. Defective EPS- or LPS-structures might result in enhanced plant defense responses.

At different steps of nodule organogenesis, the host plant can rapidly switch to defense responses, suggesting that there exist more than one control mechanism discriminating between a symbiotic and a parasitic type of development. The rhizobia, on the other side, have evolved strategies to overcome these plant defensive barriers. For instance, soybean rhizobia have been shown to possess an inducible resistance against glyceollin (Parniske et al. 1991a). Another example for rhizobial strategies to overcome plant defense responses might be the inhibition of plant enzymes by rhizobial EPS, as has been reported by Ahlborn and Werner (1991). They found that the callose synthesizing enzyme (glucan-synthase II) from G. max and Pisum sativum is specifically inhibited by the addition of wild-type rhizobial exopolysaccharide preparations, whereas surface polysaccharides of different non-rhizobial origins did not perform inhibitory effects.

Recently, Niehaus et al. (1993) reported on plant defense and delayed infection of alfalfa nodules induced by a R. meliloti exoY mutant. Although this refers to an indeterminate nodule type and, despite the fact that the mutation was within another gene which putatively encodes for a membrane associated protein homologous to hexose transferases (Müller et al. 1993), in both systems delayed infections and various indications for plant defense reactions were observed due to defects in the synthesis of extracellular polysaccharides of the inoculant strains. In the combination G. max and B. japonicum exoB mutant ΔP22 these effects were less pronounced compared to the combinations of M. sativum/R. meliloti exoY mutant or G. soja/B. japonicum exoB mutant. Furthermore, in both systems, after a prolonged incubation time, the EPS-deficient mutants were able to infect the plant tissue and to evoke the formation of effective nodules. The use of other host plants like Macroptilium atropurpureum in combination with the B. japonicum exoB mutant ΔP22 resulted in the formation of necrotic areas within the central nodule tissue (K. Kosch, unpublished).

Based on our observations we propose that intact EPS of B. japonicum is necessary to prevent elicitation of plant defense responses in soybean root cells. This working hypothesis explains why B. japonicum exoB mutants with a defective EPS were less competitive (Parniske et al. 1993) and induced early defense responses in the prospective host plant. Specific rhizobial EPS structures appear to be involved in preventing plant defense responses, at least in the early stages of the symbiosis. Once the bacteria are enclosed by the infection thread, they lose most of their EPS coat and down-regulate their EPS production (Tully and Terry 1985). In this situation, the outer membrane is no longer masked by capsular polysaccharides and contact between rhizobial LPS and the plant plasma membrane is thought to occur (Kijne 1992). LPS most probably substitutes for EPS with respect to suppression of plant defense reactions at this stage of the interaction. This idea is consistent with the crucial role of intact LPS in the infection process of soybean reported by Stacey et al. (1991). They found that specific LPS mutants of B. japonicum were unable to infect the host cells. Further evidence is presented by the work of Perotto et al. (1994) about lipopolysaccharide-defective mutants of R. leguminosarum which induce a host defense response in pea nodules. As a result, cell and tissue invasion is reduced. The mechanisms by which EPS suppresses defense responses are still unknown. Basically there are two possibilities. The wild-type EPS might prevent plant defense reactions or alternatively, a structurally modified EPS produced by an EPS-defective (brady)rhizobial strain could elicit plant defense reactions. This working hypothesis implies the possibility that, as in R. meliloti, the biological function of EPS can be associated with a single active low molecular weight fraction of the entire EPS (Battisti et al. 1992). In this respect, the results of K. Miller et al. (1994) that cyclic β-1,6-1,3 glucans from B. japonicum USDA 110 elicit glyceollin and daidzein production in soybean cotyledons are of special interest. The focus of our future investigations...
will be an attempt to answer these questions. This should lead to a better understanding of the prerequisites for the infection of plants not only by symbiotic but also by pathogenic bacteria.

MATERIALS AND METHODS

**exoB mutants of *B. japonicum***.

The strain *B. japonicum* 110osp4 was used as the wild-type strain (Hahn and Hennecke 1984). The construction of *B. japonicum* exoB mutants was as described previously (Parniske et al. 1993).

**Growth of plants.**

*G. max* ‘Preston’ (Pioneer Hi-Bred International Inc., IA) was grown in growth pouches or Leonard jars as previously described (Parniske et al. 1993). *G. soja* PI468397 was surface sterilized by immersion in 30% *H₂O₂* for 10 min, washed 10 times with sterile water, imbibed with water for 4 hr, and germinated on LN-agar for 3 days in a growth cabinet (for conditions see Parniske et al. 1993). The seedlings were transferred to petri dishes (15 cm diameter) containing nitrogen-free nutrient solution (LN-agar, Broughton and Dilworth 1971), solidified with 8 g l⁻¹ gelrite (Fa. Roth, Karlsruhe, Germany). For infection of the seedlings, the surface of the gelrite-plates were streaked with 0.5 ml of a suspension of *B. japonicum* cells (approx. 10⁷ cfu·ml⁻¹) grown in SMM (Schmidt et al. 1992).

**Light microscopy.**

For light microscopy, specimens were fixed in 2% glutaraldehyde buffered with 50 mM potassium phosphate (KPP), pH 7, for 2 hr, washed twice with KPP, followed by an increasing ethanol series of 25, 50, 75, and 96% ethanol (1 hr each). Embedding was performed in 50% LR White (London Resin Co. Ltd., UK) for 16 hr followed by 100% LR White for at least 4 days with one change of resin. The resin was polymerized at room temperature by the addition of 1% (v/v) acryl accelerator. Thin sections (1–3 μm) were cut using a microtome equipped with a glass knife. Sections were stained with toluidine blue and observed by bright field microscopy.

**Analysis of glyceollin.**

For the analysis of soybean seedling root exudate, *G. max* ‘Preston’ seeds were surface sterilized for 10 min in 30% *H₂O₂*, washed 10 times with sterile *H₂O* and then soaked for 6 hr in sterile *H₂O*. Seedlings were germinated on LN-agar for 2 days at 28°C in the dark. Roots of 2-day-old soybean seedlings were transferred to sterile 2.3-ml test tubes containing cellulose acetate filter strips (0.5 × 6.5 cm, Schleicher & Schüll, Göttingen, Germany). For infection of the seedlings, the surface of the gelrite-plates were streaked with 0.5 ml of a suspension of *B. japonicum* cells (approx. 10⁷ cfu·ml⁻¹) grown in SMM (Schmidt et al. 1992).

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For light microscopy, specimens were fixed in 2% glutaraldehyde buffered with 50 mM potassium phosphate (KPP), pH 7, for 2 hr, washed twice with KPP, followed by an increasing ethanol series of 25, 50, 75, and 96% ethanol (1 hr each). Embedding was performed in 50% LR White (London Resin Co. Ltd., UK) for 16 hr followed by 100% LR White for at least 4 days with one change of resin. The resin was polymerized at room temperature by the addition of 1% (v/v) acryl accelerator. Thin sections (1–3 μm) were cut using a microtome equipped with a glass knife. Sections were stained with toluidine blue and observed by bright field microscopy.

**Analysis of glyceollin.**

For the analysis of soybean seedling root exudate, *G. max* ‘Preston’ seeds were surface sterilized for 10 min in 30% *H₂O₂*, washed 10 times with sterile *H₂O* and then soaked for 6 hr in sterile *H₂O*. Seedlings were germinated on LN-agar for 2 days at 28°C in the dark. Roots of 2-day-old soybean seedlings were transferred to sterile 2.3-ml test tubes containing cellulose acetate filter strips (0.5 × 6.5 cm, Schleicher & Schüll, Göttingen, Germany), submerged in either moropholino ethane sulfonic acid (MES) buffer (5 mM, pH 6.2) or cellulose acetate filter Strips (0.5 x 6.5 cm, Schleicher & Schüll, Göttingen, Germany). For infection of the seedlings, the surface of the gelrite-plates were streaked with 0.5 ml of a suspension of *B. japonicum* cells (approx. 10⁷ cfu·ml⁻¹) grown in SMM (Schmidt et al. 1992).

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**LITERATURE CITED**


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