Research Articles

Phytoalexin response is elicited by a pathogen (*Rhizoctonia solani*) but not by a mycorrhizal fungus (*Glomus mosseae*) in soybean roots

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Received 11 September 1990; accepted 15 October 1990

Summary. A container system was constructed to study the response of soybean roots to infection by mycorrhizal or pathogenic fungi. The system allows a rapid and synchronous inoculation by *Glomus mosseae* or *Rhizoctonia solani*. The phytoalexin glyceollin was measured in roots of inoculated and uninoculated plants for a period of 30 days. A significantly increased content of phytoalexin was found in *R. solani*-infected roots as compared to uninfected control roots. However, there was no difference in the glyceollin contents of the mycorrhizal and the control roots for up to 23 days after inoculation. The accumulation of glyceollin in *R. solani*-infected roots was not influenced by a subsequent inoculation with *G. mosseae*. Moreover glyceollin accumulated in mycorrhizal plants to the same extent as in control plants when they were inoculated with *R. solani*. The two fungi did not mutually influence the course of infection when they were inoculated together.
Key words. VA-mycorrhiza; *Glycine max*; glyceollin; rhizosphere; rhizotron.

The vesicular-arbuscular mycorrhiza (VAM) is a widespread symbiosis between plants and fungi. Although there are many studies on the established symbiosis, for instance concerning phosphorus uptake, carbon transport, heavy-metal toxicity or the influence of pesticides, little is known about the physiology of the interaction between plant and fungus at the beginning of infection [1]. In particular it is poorly known whether the mutualistic character of the established symbiosis manifests itself already at the beginning of infection, and whether recognition differs from that involved in pathogenic interactions.

One reason for the lack of data is the difficulty of handling mycorrhizas in the laboratory. A fast and homogeneous course of infection is needed. In the present work we have employed a modified version of the container system of Schüepp et al. [2] which allows one to obtain such infections and therefore to investigate the first infection stages of mycorrhiza formation. A short description of our technique has already been published [3].

We have used this system to compare the phytoalexin response of roots to infection by a VAM fungus and a pathogen. Phytoalexins are antimicrobial compounds of low molecular weight which accumulate in plants after exposure to microorganisms. Their production appears to result from de novo synthesis of the corresponding biosynthetic enzymes upon a challenge of the plant by a pathogen or an elicitor [4, 5]. There is evidence that accumulation of phytoalexins at the site of attempted infection is one of the mechanisms conferring resistance against infections in plants [6]. The phytoalexin response to pathogens has been particularly well studied in soybeans which accumulate the pterocarpan glyceollin [5]. Little is known about the accumulation of this phytoalexin in response to mycorrhizal fungi. An early report indicated glyceollin accumulation in the fully established VAM symbiosis [7]. Using our container system, we have recently obtained first indications that the phytoalexin response does not come into play during the early stages of mycorrhizal infection [3]. Our results now extend these observations, demonstrating that soybean roots do not accumulate phytoalexins in response to mycorrhizal fungi and during the establishment of the VAM symbiosis. The accumulation of phytoalexin in response to a pathogen is not influenced by the mycorrhizal fungi.

Materials and methods

System for inoculation. To achieve a fast and homogeneous infection a special system has been developed which allows uninfected test plant roots on one side of a nylon net to be brought into close contact with heavily infected inoculum plant roots on the other side. The system consists of two types of PVC containers (see fig. 1):

1) inoculum plant containers with the inoculum plants with well-established mycorrhizal or pathogen-infected roots (non-infected plants are used as control inocula), and 2) test plant containers with five compartments in each of which there is a test plant grown in sterile soil. The compartments can be opened separately for sampling by pulling up the closing lid. After sampling the closing lid is put back into the compartment to prevent the drying-out of the inoculum plant soil.

The two types of containers can be joined or separated at any time without disturbing the root systems since the

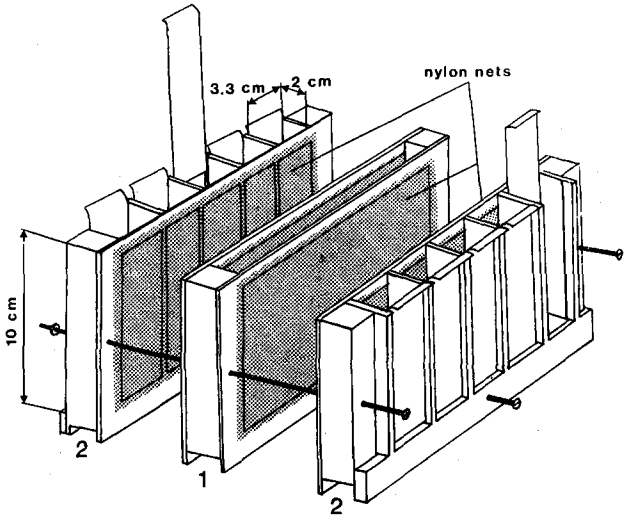

Figure 1. Containers for plant root inoculation: 1) Inoculum plant container in which well-established mycorrhizal, pathogen-infected, or non-infected plants are grown. 2) Test plant container with five compartments in which single test plants are grown in sterilized soil.

test plant roots and the inoculum plant roots are separated by nylon screens (60 µm mesh, mounted with tetrahydrofuran on both types of containers) which can be penetrated by fungal hyphae but not by roots.

Biological material. All plants were grown in a greenhouse (day/night cycle: 14 h, 27 °C/10 h, 20 °C) in a steam-sterilized (20 min, 121 °C) mixture of sand, loam and organic matter (3:2:1) with a pH ($CaCl_2$) of 7.6. Steam-sterilized wheat seeds were inoculated with *Rhizoctonia solani* Kühn (*Thanatephorus cucumeris* (Frank) Donk) and kept for two weeks at room temperature in the dark. Pathogen-infected inoculum plants, soybean (*Glycine max* L. Maple Arrow) and white clover (*Trifolium repens* L. cv Neuseeländer), were produced by planting 5-day old seedlings into a sterilized soil mixture mixed with *R. solani*-infected wheat seeds (2.5% v/v), one to five weeks before the experiment. Mycorrhizal inoculum plants were similarly inoculated four to eight weeks before the experiment with spores and mycelium of *Glomus mosseae* ((Nicol. & Gerd.) Gerd. & Trappe), obtained from mycorrhizal stock cultures on expanded clay[8]. Control inoculum plants were mock-inoculated with sterile expanded clay at the corresponding time.
Seeds of test plants (*G. max* L. Maple Arrow) were surface-sterilized by soaking in 0.75% sodium hypochlorite for 5 min, rinsed with tap water and germinated in Vermiculite. Seedlings of homogeneous size were planted into the test plant containers after three days of germination. Test plant containers were joined to the inoculum plant containers five days later. Duplicates of test plants were always harvested and processed separately. In the first series of experiments, samples were taken at intervals of 2–4 days from day 0 to day 30 after inoculation. In the second series of experiments, samples were taken 7 and 15 days after inoculation. At day 15, the test plant containers were separated from the inoculum plant containers and joined with other inoculum plant containers holding either the same or a different type of inoculum. Samples were then taken for another 15 days at intervals of 2–4 days.

Quantitation of glyceollin. Roots were sampled, washed and cut into 1–2 cm segments. After determination of the fresh weight they were stored in 95% ethanol at −20 °C until used. Glyceollin was extracted with 95% ethanol (10 ml g^{-1} f.wt) 2 × 36 h at room temperature. The ethanol was evaporated to dryness at 35 °C under a stream of N_2. The residues were extracted twice in 700 µl and 500 µl of ethyl acetate, respectively. The combined extracts were transferred to Eppendorf tubes and the solvent removed under partial vacuum. The residues were taken up in 50 µl of ethyl acetate and subjected to TLC (Whatman LK6DF silica gel plates, 0.25 mm) using chloroform/acetone/NH_4OH (25%) (50:50:1) as a solvent[9]. Glyceollin was located on the TLC plates by comparison with a co-chromatographed standard (see below), using fluorescence quenching in UV light (254 nm) for detection. Glyceollin-containing bands were scraped from the plates and eluted with 2 ml of ethanol. After centrifugation, the supernatant was evaporated to dryness at 35 °C under a stream of N_2. The residue was taken up in 1 ml of ethyl acetate, transferred to Eppendorf tubes and dried under partial vacuum. After redissolving in 100 µl methanol 100% and filtering (Millipore HV 0.445 µm) the glyceollin was then measured by its absorption at 285 nm upon separation by HPLC on a reversed phase column (Hypersil ODS 5 µm, 250 × 4.6 mm) with 60% (v/v) methanol as the mobile phase (flow rate 1 ml min^{-1}). The three isomers of glyceollin (I, II, III) were separated and were found to occur in a ratio of 15:1:1. The values given correspond to the sum of the three isomers.
For reference, glyceollin standards were prepared by inducing phytoalexin accumulation in wounded cotyledons from soybean with *Nectria haematococca* Berk. & Br. spores. Extraction and purification was carried out as described above. Glyceollin was localized on TLC by the *Cladosporium cucumerinum* Ell. & Arth. bioassay[10] and purified by HPLC as described above. The standard sample was 99% pure in a HPLC run and showed the expected absorption maximum at 285 nm.

Estimation of root infection. The root segments from which glyceollin had been extracted were cleared and stained after the method of Phillips and Hayman[11] but without the phenolic component in the solvent agent. Estimations of root infection by *G. mosseae* and *R. solani* were made by the gridline intersect method[12]. Data are given as percentages of root length infected.

Statistical analysis. Twelve repetitions of experiment 1 were included in a statistical analysis. The glyceollin values were transformed logarithmically. The data were grouped into eight classes: 0–2, 3–5, 6–8, 9–11, 12–14, 15–18, 19–23 and 24–30 days after inoculation. Multiple regression analyses were then carried out (GENSTAT statistical program). The following factors were included in the model: degree of infection, treatment, treatment-infection interaction, time class, repetition-replicate-treatment interaction. This last term was included to calculate adjusted means, by subtracting from each data point the effect due to the particular experimental run (one series of data points over time). At the end of the analysis the values were backtransformed from a logarithmic to the original scale.

Results

Inoculation and infection course. Test plants were rapidly infected after joining their containers to inoculum plant containers holding mycorrhizal clover or soybean (fig. 2). The first mycorrhizal structures – intraradical hyphae and arbuscules – were already observed in the test plants three days after inoculation. The degree of infection increased continually up to 23 days after inoculation, when about 71 % of the total root length was infected. Test plants joined to the inoculum plant containers holding plants infected with *R. solani* showed the first infection structures, consisting of infection cushions and mycelium on the root surface, within two days. *R. solani* was rarely able to penetrate the epidermis and to grow intraradically, a typical feature of an incompatible plant-fungus interaction. Maximum levels of infection – 31 % of root length infected – were observed between days 9 and 14 after inoculation. Thereafter the degree of infection decreased slightly but steadily to 22 %.

Twelve repetitions of this inoculation experiment showed the same temporal pattern. The absolute values of infection levels varied somewhat but the time course was always similar. In particular, the time course of infection never varied significantly ($p = 0.21$) in the replicates.

In a second series of experiments, the inoculum plant containers were changed after 15 days. In test plants initially infected with *G. mosseae* and transferred to a new inoculum of *G. mosseae* after 15 days, the degree of infection remained constant for 4 days and thereafter increased again (fig. 4A). Similar test plants, preinoculated with *G. mosseae* but transferred to a control or an inoculum of *R. solani* after 15 days, showed a similar pattern (fig. 4A), indicating that the further increase in mycorrhizal infection after the exchange of inoculum was due to spreading of the mycorrhizal infection within the test plant containers. Test plants pre-inoculated with a *R. solani* inoculum or a control and transferred to a *G. mosseae* inoculum after 15 days showed a comparable time course of infection by the mycorrhizal fungus (fig. 4B).

The course of infection by *R. solani* in plants pre-inoculated with *R. solani* and transferred to a control or to inocula of *G. mosseae* or *R. solani* after 15 days showed the same pattern in all variations (fig. 4C). There was an increase of infection up to day 19 after inoculation and a decrease afterwards. Plants pre-inoculated with *G. mosseae* or control plants transferred to *R. solani* inoculum after 15 days were comparable with respect to the course of infection by *R. solani* (fig. 4D).

Accumulation of glyceollin. In mock-inoculated plants there was virtually no accumulation of glyceollin whereas in *R. solani*-inoculated plants glyceollin began to accumulate as soon as they came into contact with the fungus (fig. 3). In contrast, mycorrhizal plants accumulated no

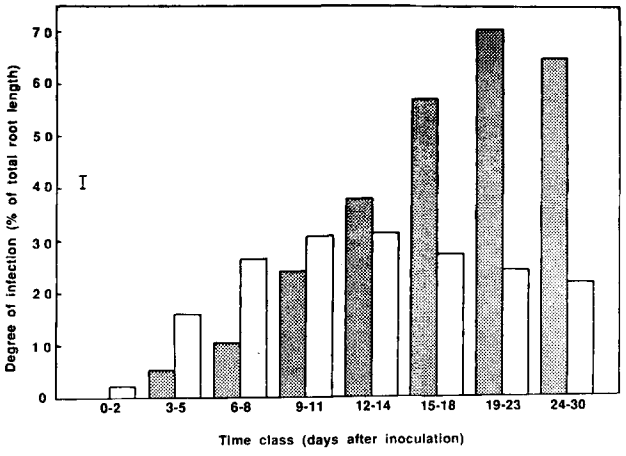

Figure 2. Time course of infection of soybean roots by *Glomus mosseae* (▨) and *Rhizoctonia solani* (□). Statistical analysis of the values of 12 repetitions, each with two replicates per treatment (I: standard error of difference).

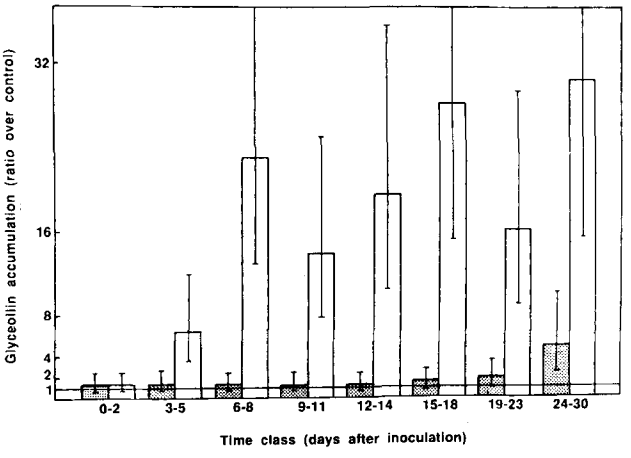

Figure 3. Glyceollin levels in soybean roots infected with *Glomus mosseae* (▨) and *Rhizoctonia solani* (□), expressed as the ratio between glyceollin levels in infected and non-infected roots of the same time class. Statistical analysis of the values of 12 repetitions, each with two replicates per treatment. The values were logarithmically transformed for statistical analysis and backtransformed to the original scale for expression of results. Each column represents the mean ratio with 95 % confidence limits. Non-infected roots contained 0.01–0.06 µg glyceollin per g f.wt and the maximal level reached after infection by *R. solani* was in the range of 37 µg per g f.wt.

glyceollin during the first 3 weeks of infection. Up to 23 days after inoculation, the extremely low levels of glyceollin detected in mycorrhizal plants were statistically indistinguishable ($p > 0.25$) from the level in control plants. An accumulation of small amounts of glyceollin occurred 24 days after inoculation in the mycorrhizal plants.

The interaction of sequential infections by different fungi (see fig. 4) with regard to glyceollin accumulation was also tested (fig. 5). In plants which had been in contact only with the mycorrhizal inoculum or with the control inoculum, glyceollin accumulation was very low over the whole experiment (fig. 5A and 5B). In contrast, glyceollin accumulation was highly induced within 15 days in plants inoculated with *R. solani* (fig. 5C), in accordance with the finding shown in figure 3. The type of inoculum present during the second period of 15 days did not significantly influence the pattern. Roots first inoculated with *G. mosseae* (fig. 5B) or with control inoculum (fig. 5A) accumulated glyceollin in comparable amounts as soon as they came in contact with *R. solani*.

Discussion

The container system described allows a rapid and synchronous infection of test plant roots by a mycorrhizal or a pathogenic fungus. The test plant roots can be sampled easily throughout the course of infection without disturbing the other roots.

Infection of the roots by *R. solani* rapidly induced accumulation of glyceollin. This is in accordance with the observation in incompatible pathogenic systems[4,6]. In contrast, from the early stages of root colonization by the mycorrhizal fungus *G. mosseae* until the stage of maximal level of infection, there was virtually no accumulation of glyceollin in soybean roots. Only a slight accumulation of glyceollin was observed at a late stage of infection, as has already been described by Morandi et al.[7].

In soybean plants pre-inoculated with *G. mosseae* and then inoculated with *R. solani*, the amount of glyceollin accumulated, and the time course of accumulation, were similar to those observed for plants inoculated only with *R. solani*. Moreover, glyceollin accumulation was not influenced by *G. mosseae* in plants which had been pre-inoculated with *R. solani*. These results suggest that the absence of glyceollin accumulation in mycorrhizal roots is due to a lack of induction of the enzymes involved in glyceollin biosynthesis, rather than to a suppression of these enzymes, or to a phytoalexin-catabolizing or detoxifying activity of the mycorrhizal fungus. Interestingly, no accumulation of glyceollin was found in response to *G. mosseae* even under conditions of high P-supply, when the mycorrhizal symbiosis is fully suppressed[13]. The lack of phytoalexin accumulation in response to the mycorrhizal fungus is comparable to the lack of host defense reactions in response to biotrophic pathogens well adapted to their host and growing through the plant tissue without causing apparent damage to the host until the pathogen has colonized a large area of the plant[6,14]. Another symbiont of soybean, *Bradyrhizobium japonicum*, also invades the roots without inducing phytoalexin accumulation[15]. The similarity of these three types of plant-microorganism interactions lies not in the final outcome of the interaction for the host but in their biotrophic nature. In fact, there are fix$^-$ mutants of *Bradyrhizobium japonicum* which enter the roots but cause necrosis and simultaneously phytoalexin accumulation[15].

Similarities between the nodule and VAM symbiosis seem to exist with respect to active responses of the plant: mycorrhizal roots have been found to produce proteins immunologically related to nodulins, i.e. proteins specifically formed by the plants in the nodules[16]. In spite of these similarities it is unlikely that the plant recognizes

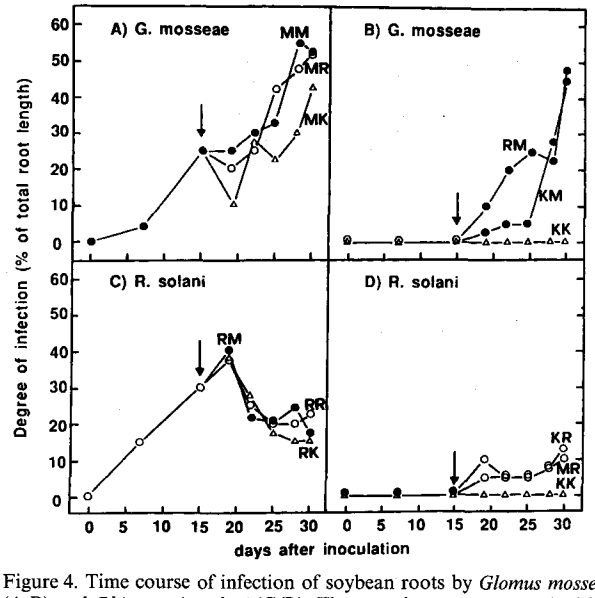

Figure 4. Time course of infection of soybean roots by *Glomus mosseae* (A,B) and *Rhizoctonia solani* (C,D). The test plants were treated with a first inoculum for 15 days and then exposed to a second inoculum (arrow), taking all possible permutations into account. The two-letter symbols designate the two sequential inocula used: *Glomus mosseae* (M), *Rhizoctonia solani* (R) and control (K).

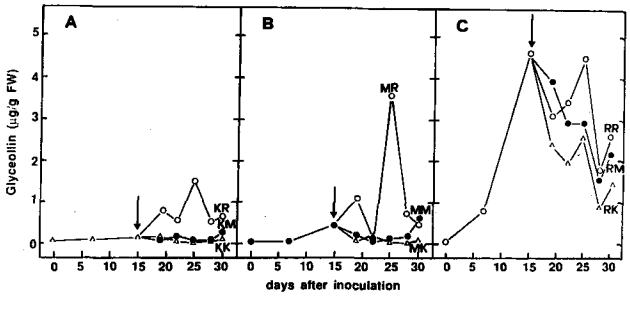

Figure 5. Glyceollin accumulation in soybean roots first inoculated with control (A), *Glomus mosseae* (B) or *Rhizoctonia solani* (C) for 15 days and subsequently exposed to a new inoculum (arrow), taking all possible permutations into account. The two letter symbols have the same meaning as in figure 4.

the mycorrhizal fungus and the rhizobium by the same mechanism. The control of the degree of infection of the root under high nutrient supply has been found to differ in these two types of symbioses [17].

A particularly intriguing aspect of the biotrophic interactions of soybean roots with microorganisms is the fact that biotrophic pathogens and nodule-forming bacteria generally display a very limited host range, often extending only to a few genotypes of a single plant species [6, 18], whereas VAM fungi generally have an extremely wide host range [1]. Further investigations will have to examine whether the lack of phytoalexin elicitation has a similar basis in the interactions of plants with biotrophic pathogens, nodule-forming bacteria and VAM fungi or whether the plants have the capability of specifically recognizing mutualistic symbionts. It may be speculated that the VAM symbiosis represents a basically compatible plant-microorganism interaction, while both nodule symbiosis and the interaction with biotrophic pathogens exemplify specialized adaptations of the partners to originally incompatible plant-microorganism interactions.

Acknowledgments. We thank Bernhard Schmid for his help in statistical analysis. This research was supported by the Swiss National Science Foundation.

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Correction

T. H. Jukes: Genetic Code 1990. Outlook; **Experientia 46 (1990) 1149–1157.**

Data in Table 3 should read as follows:

Caption: COX III should be **COX II**
Site 129: *UUG* should be ***UGG***
First column: RNA ONE should be **RNA OBE**

Furthermore, lines 38–43, left column, p. 1153, should read:

Leinfelder et al.[26] have suggested that 'UGA was originally a **sense** codon' (presumably for SeCys) and 'after introduction of oxygen into the biosphere this highly oxidizable amino acid could be maintained only in anaerobic organisms or in aerobic systems which evolved special protective mechanisms'...