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RESEARCH PAPER

Suppression of arbuscular mycorrhizal colonization and nodulation in split-root systems of alfalfa after pre-inoculation and treatment with Nod factors

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Abstract

Roots of legumes establish symbiosis with arbuscular mycorrhizal fungi (AMF) and nodule-inducing rhizobia. The existing nodules systemically suppress subsequent nodule formation in other parts of the root, a phenomenon termed autoregulation. Similarly, mycorrhizal roots reduce further AMF colonization on other parts of the root system. In this work, split-root systems of alfalfa (*Medicago sativa*) were used to study the autoregulation of symbiosis with *Sinorhizobium meliloti* and the mycorrhizal fungus *Glomus mosseae*. It is shown that nodulation systemically influences AMF root colonization and vice versa. Nodules on one half of the split-root system suppressed subsequent AMF colonization on the other half. Conversely, root systems pre-colonized on one side by AMF exhibited reduced nodule formation on the other side. An inhibition effect was also observed with Nod factors (lipo-chito-oligosaccharides). NodSm-IV(C16:2, S) purified from *S. meliloti* systemically suppressed both nodule formation and AMF colonization. The application of Nod factors, however, did not influence the allocation of ^{14}C within the split-root system, excluding competition for carbohydrates as the regulatory mechanism. These results indicate a systemic regulatory mechanism in the rhizobial and the arbuscular mycorrhizal association, which is similar in both symbioses.

Key words: Arbuscular mycorrhiza, autoregulation, Glomales, *Medicago sativa*, Nod factors, nodulation, *Sinorhizobium meliloti*, split-root system.

Introduction

Legumes are hosts for two different types of root symbionts, nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi (AMF). The establishment of symbiosis is the result of a complex series of interactions between the symbiont and the host plant (reviewed by Hirsch and Kapulnik, 1998; Albrecht *et al*., 1999). For the infection process, the exchange of symbiotic signal molecules is required. Rhizobial bacteria generally enter roots of legumes via root-hairs and induce the formation of root nodules. One important group of rhizobial signals are Nod factors (lipo-chito-oligosaccharides) (Perret *et al*., 2000). AMF hyphae colonize the root cortex and form highly branched, bush-like structures within a host cell, the so-called arbuscules. Via its extraradical mycelium, the AMF provide the plant with nutrients, mainly phosphorus (Smith and Read, 1997). There is growing evidence that processes leading to nodule initiation and mycorrhiza are similarly regulated (reviewed by Hirsch and Kapulnik, 1998; Albrecht *et al*., 1999; Guinel and Geil, 2002; Stracke *et al*., 2002; Staehelin *et al*., 2001; Vierheilig and Piché, 2002).

From the plant's perspective, the development of a symbiotic association is a beneficial, but also a costly process. To control the number of nodules, legumes have developed negative regulatory systems to maintain

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homeostasis of nodulation. The phytohormone ethylene, for example, inhibits rhizobial infection (Penmetsa and Cook, 1997). Similarly, the existing nodules systemically inhibit subsequent nodulation in other parts of the root system. This feedback control is termed autoregulation. In split-root systems, rhizobial inoculation of one half of the root system partially blocks subsequent nodulation of the other half. Grafting experiments revealed that some forms of autoregulation are controlled by the shoot (for details see review by Caetano-Anollés and Gresshoff, 1991*a*). Recently, it has been shown that nodulation is suppressed when one side of a split-root system was pretreated with Nod factors, indicating that the observed autoregulatory effect is triggered by a pre-nodulation event (van Brussel *et al*., 2002) and a first gene involved in this autoregulation has been identified. Har1 and Nts mutants of *Lotus japonicus* and soybean, respectively, lost their ability to regulate the nodule number and thus display a super-nodulating phenotype. The mutated genes encode putative receptor kinases with similarities to *CLAVATA1* of *Arabidopsis*, which negatively regulates tissue differentiation. It has been hypothesized that the receptor kinase interacts with a small peptide involved in systemic shoot-controlled regulation of nodule development (Krusell *et al*., 2002; Nishimura *et al*., 2002; Searle *et al*., 2003).

An autoregulatory mechanism has been also described for the AMF symbiosis. In barley plants, prior mycorrhizal colonization of one side of a split-root system resulted in a suppression of further root colonization on the other side (Vierheilig *et al*., 2000*a*, *b*). Thus, systemic autoregulatory mechanisms seem to control both nodule formation and AMF colonization. In this study, it was demonstrated that autoregulation of one symbiosis systemically affects the outcome of the other symbiosis.

Materials and methods

Biological material, growing conditions and experimental design

Alfalfa (*Medicago sativa* L. cv. Sitel) plants were infected with *Glomus mosseae* (Nicolson & Gerdemann) Gerd. & Trappe (BEG 12; European Bank for the Glomales). For nodulation experiments, *Sinorhizobium meliloti* strain 1021 was used (http://sequence.toulouse.inra.fr/meliloti.html).

Corresponding control plants were grown under the same conditions. Alfalfa plants were grown in a growth chamber (day/night cycle: 16/8 h, 23/19 °C; relative humidity 50%; light: 300 µmol m^{-2} s^{-1} PAR (photosynthetically active radiation)). All experiments were repeated twice with five replicates per treatment.

Split-root systems

Alfalfa seeds were surface-sterilized by soaking in 0.75% sodium hypochlorite for 5 min, rinsed with tap water and germinated in pots in a steam-sterilized (40 min, 120 °C) mixture of silicate sand, TurFace (Applied Industrial Materials Corp.; Buffalo Grove, Illinois, USA) and soil (1:1:1 by vol.). To induce the development of lateral roots, tips of main roots from 11-d-old plantlets were cut off. Plantlets were then placed into the same substrate as described above. Four weeks later, split-root systems were established as described previously (Vierheilig *et al*., 2000*a*). Briefly, the split-root system consists of two units, each containing one half of the alfalfa root system. The two compartments are separated by an impermeable PVC membrane in order to prevent any flow of molecules or root growth from one side to the other. Thus one side of the split-root system can be inoculated with one of the symbionts or treated with Nod factors without any contact with the other side.

Inoculation with AMF fungi

The outer side of each split-root compartment is equipped with a nylon screen (60 µm mesh), which can be penetrated by hyphae but not by roots. To inoculate alfalfa with *G. mosseae,* this outer side was joined to a similarly designed compartment. This donor compartment contained beans (*Phaseolus vulgaris* L. cv. Sun Gold) colonized by *G. mosseae*. To avoid contamination of the beans in the donor compartments by rhizobia, plants were fertilized three times a week with 5 ml of a KNO_3 (0.808 g l^{-1}), $Ca(NO_3)_2.4H_2O$ (1.808 g l^{-1}) solution until 3 weeks before the experiment. No nodulation of beans could be observed throughout the experiment.

After 19 d, 53±8% of the alfalfa roots in the neighbouring compartment were colonized by the fungus. No nodules could be detected. The other half of the split-root system remained non-infected.

Inoculation with Sinorhizobium meliloti

Rhizobium strain 1021 was cultivated on TY-streptomycin (50 µg ml^{-1}) media at 25 °C on a rotary shaker (200 rpm) to reach an OD_{600}=0.2. Cells were then harvested by centrifugation (6000 *g* for 10 min) and resuspended in sterile H_2O. For inoculation, 5 ml of this bacterial suspension was added to a selected compartment of the split-root system. After 15 d, alfafa roots formed 48±9 nodules (per 100 mg root FW), whereas the other compartment of the split-root system remained without nodules.

Application of Nod factors to roots

The tetrameric Nod factor NodSm-IV(C16:2, S) was purified from *S. meliloti* strain 1021(pEK327) (Schultze *et al*., 1992). Supernatants of bacterial cultures were extracted with *n*-butanol and fractionated by reverse-phase HPLC (Waters C18 column), using 35% acetonitrile/water and 40 mM ammonium acetate as the mobile phase. The fraction containing NodSm-IV(C16:2, S) was desalted as described by Staehelin *et al*. (2000). Four ml of a 10^{-8} M solution (dissolved in water) were daily applied to one side of the split-root system.

Treatment with $^{14}CO_2$

For the $^{14}CO_2$ labelling experiment, plant shoots were placed inside a 945 ml (18×20 cm) transparent freezer bag (Ziploc®) together with a 29.5 ml cup containing a basic solution of 37 kBq (1 µCi) $NaH^{14}CO_3$ (Amersham Pharmacia Biotech). The plastic bag was then closed and a sealing compound placed around the shoot stem. Gaseous $^{14}CO_2$ was produced by injecting 1 ml lactic acid (85%) into the cup.

Determination of nodule number and AMF root colonization

At the time of harvest, roots were carefully rinsed with water and the root fresh weight and the number of nodules were determined. To estimate AMF root colonization, several roots from each plant were cleared by boiling in 10% KOH and stained according to the method of Vierheilig *et al*. (1998) by boiling in a 5% ink (Shaeffer; black)/household vinegar (=5% acetic acid) solution. Stained roots were observed with a light microscope to determine the percentage of root colonization according to a modified method of Newman (1966).

AMF colonization followed by subsequent AMF colonization and nodulation

After the transfer of plants into a split-root system, one compartment was immediately inoculated with the AMF. After 19 d, when the AMF was well established (Vierheilig *et al*., 2000*a*, *b*), the second compartment was inoculated either with AMF or with *S. meliloti*. Plants colonized by AMF were harvested 11 d later. Plants inoculated with *S. meliloti* were harvested 14 days post-inoculation (dpi).

Prior nodulation followed by subsequent nodulation and AMF colonization

Four days after establishment of the split-root system, the first compartment was inoculated with *S. meliloti.* After 15 d, the second compartment was inoculated either with *S. meliloti* or with AMF. The plants inoculated with AMF were harvested 11 d later. Plants infected with *S. meliloti* were harvested 14 d after inoculation of the second compartment.

Effect of Nod factors on nodulation and AMF colonization

Eleven days after establishment of the split-root system, Nod factors were applied to one half of the split-root system. This treatment was repeated daily until the time of harvest. Eight days after the first treatment, the second compartment was inoculated either with AMF or with *S. meliloti.* The mycorrhizal roots were harvested 11 dpi. and the nodule number was determined 14 dpi.

^{14}C allocation in a split-root system after treatment with Nod factors

Eleven days after establishing the split-root system, Nod factors were applied to one half of the root system. Eight days later, plant shoots were exposed to $^{14}CO_2$ (see above) and incubated for 2 h in the growth chamber. After this pulse period, the bags were removed under a venting fume-hood and plants were returned to the growth chamber. After a 24 h chase period, the two halves of the split-root system were harvested and their fresh weights recorded. The root systems were then oven-dried (24 h at 65 °C), weighed, and used to determine the level of incorporated radioactivity. Roots were ground in liquid nitrogen and digested according to the technique described by Clifford *et al*. (1973) with the tissue solubilizer NCS. Radioactivity was assessed by liquid scintillation spectrometry. Counts were standardized with a quench curve and expressed in dpm. Data were indicated as percentage of ^{14}C in the total root.

Results

Effects of either AMF or Sinorhizobium *on subsequent AMF colonization*

Split-root systems are a useful tool to study autoregulation, i.e. systemic suppression of subsequent root colonization by an already colonized part of the root. In this work, alfalfa plants were used, which have been reported to exhibit strong autoregulation of nodule formation (Caetano-Anollés and Bauer, 1988; Caetano-Anollés and Gresshoff, 1991*b*). After cutting off the tip of the main root, plants developed lateral roots within a relatively short time and formed a homogeneous root system, which was separated into two equal parts. It was investigated in a first experiment whether pre-inoculation of one side of the root system influenced subsequent AMF colonization on the other part. The first half of the split-root system was either inoculated with the mycorrhizal fungus *G. mosseae* or with nodule inducing *S. meliloti*. Control plants were not inoculated on this side of the split-root system. After 19 d, all plants were inoculated with *G. mosseae* on the second half of the split-root system. 11 d later, AMF colonization was investigated on harvested roots. AMF colonization in the pre-inoculated side was 57±6% and plants infected with *S. meliloti* formed 107±11 nodules (24±8 nodules per 100 mg root FW) on this half of the root system. As seen in Fig. 1, the degree of AMF colonization in the second side of the split-root system depended on the treatment of the first side. Compared to control plants without pre-inoculation, subsequent AMF colonization was reduced in plants already colonized by *G. mosseae.* Interestingly, a similar reduction of AMF colonization was observed when the first half of the split-root system was infected with *S. meliloti*. These data show that already formed nodules on one part of the root system inhibit subsequent AMF colonization of other parts of the root system.

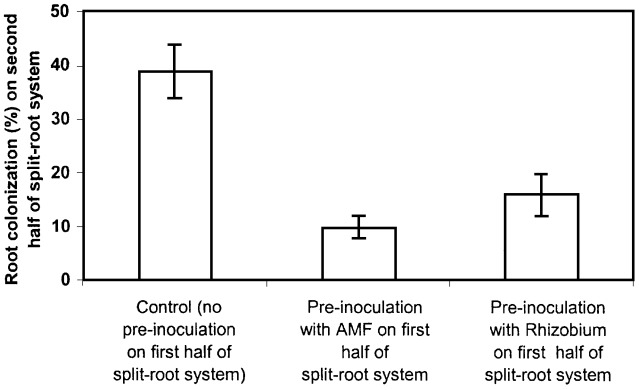

Fig. 1. Effect of pre-inoculation on subsequent AMF colonization in alfalfa roots. Data (means ±SE) indicate the degree of mycorrhizal colonization (expressed as % of total root length) in the second compartment of the split-root system. The first compartment was pre-inoculated either with *G. mosseae* (root colonization at the time of harvest 57±6%) or with *S. meliloti* (107±11 nodules). Control plants were not pre-inoculated.

Effects of either AMF or Sinorhizobium *on subsequent nodule formation*

In a reciprocal experiment, it was tested whether the establishment of symbiosis on one compartment of the split-root system inhibits subsequent nodule formation in the other compartment (Fig. 2). Control plants were not pre-inoculated and formed 263±33 nodules on the inoculated part of the split-root system. Nodules in the first compartment strongly suppressed subsequent total nodulation (Fig. 2A) and number of nodules per 100 mg root FW (Fig. 2B), indicating autoregulation of nodule formation. A suppression of nodule development was also observed when plants were inoculated with AMF on one side of the root system and subsequently infected with *S. meliloti* on

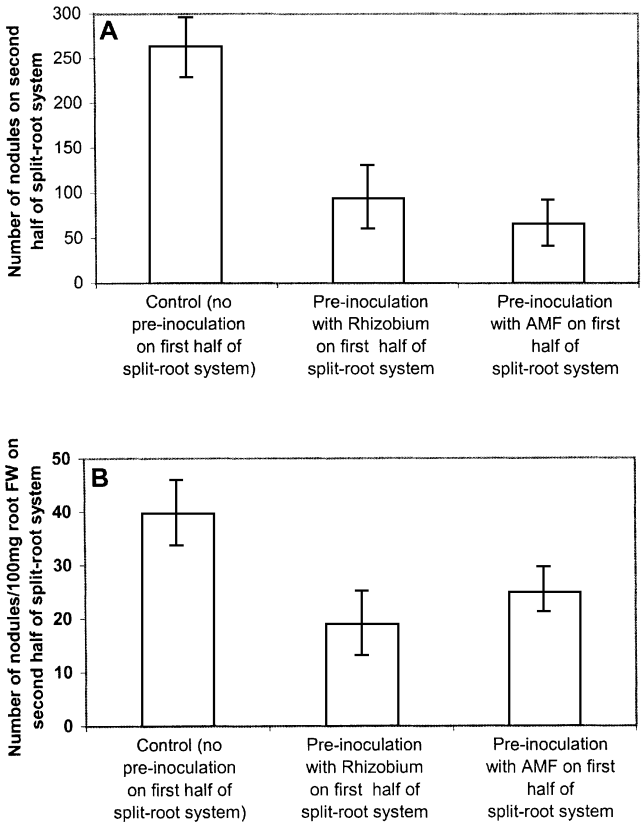

Fig. 2. Effect of pre-inoculation on subsequent nodule formation. Data (means ±SE) indicate the number of nodules (A) and the number of nodules per 100 mg root FW (B) in the second compartment of the split-root system. The first half of the split-root system was pre-inoculated either with *G. mosseae* (root colonization at the time of harvest 63±3%) or with *S. meliloti* (228±46 nodules). Control plants were not pre-inoculated.

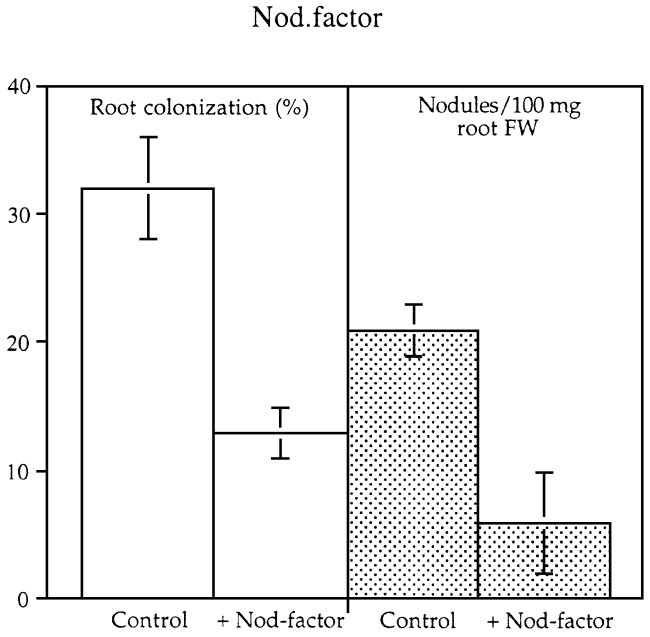

Fig. 3. Application of Nod factors suppresses AMF colonization and nodule formation in split-root systems. The first compartment was daily treated with 4 ml of 10^{-8} M NodSm-IV(C16:2, S). Control plants were treated with 4 ml water. Eight days later, the second compartment was inoculated either with *G. mosseae* or with *S. meliloti.* Data (means ±SE) indicate the degree of AMF root colonization (left panel) and the number of formed nodules per 100 mg root FW (right panel) at the time of harvest.

the other side. When looking at the total nodule number, these plants formed approximately 4-fold fewer nodules compared with control plants which were not pre-inoculated on the first half of the root system (Fig. 2A). Similar data were obtained when root FW was used as the basis. The nodule number per 100 mg root FW was suppressed by an already established symbiotic interaction (Fig. 2B). These data indicate that AMF colonization of one side of the root system inhibited nodulation on the other side.

Systemic effects of Nod factors on nodule formation and AMF colonization

S. meliloti produces a number of Nod factors, which trigger early host plant responses that enable bacterial entry into root-hairs. To test the effect of purified Nod factors on the subsequent establishment of symbiosis, the first half of the split-root system was treated with 10^{-8} M NodSm-IV(C16 :2, S), a tetrameric sulphated Nod factor from *S. meliloti*. The application was repeated daily until plants were harvested. As seen in Fig. 3, the application of NodSm-IV(C16 :2, S) on one side of the split-root system inhibited nodule development on the other side, indicating an autoregulatory feedback response. Moreover, treatment of roots with NodSm-IV(C16 :2, S) also inhibited AMF colonization. Mycorrhizal colonization on the second part of the split-root system was approximately 2-fold lower compared with control plants which were not treated with Nod factors.

Carbon partitioning in split-root systems after application of Nod factors

To test the effect of Nod factors on carbon partitioning, NodSm-IV(C16 :2, S) was applied to one half of the split-root system and the leaves were treated with $^{14}CO_2$. Incorporation of ^{14}C was separately measured for the two compartments of the split-root system. Application of NodSm-IV(C16 :2, S) to one half of the split-root system did not affect carbon allocation. The carbon sink strength of the side treated with Nod factors (^{14}C=51±6%) was similar to the non-treated side (^{14}C=49±14%).

Discussion

Establishment of symbiosis on one part of the root thwarts further microbial colonization on other parts of the root system. This systemic feedback-control, termed auto-

regulation, has been described for nodule formation of legumes (Caetano-Anollés and Gresshoff, 1991*a*) and AMF colonization (Vierheilig *et al*., 2000*a*, *b*). It has been suggested that plants evolved autoregulatory mechanisms to limit the costs for establishment of symbiosis (Caetano-Anollés and Gresshoff, 1991*a*; Vierheilig and Piché, 2002). This study shows that precolonization of roots with AMF not only systemically inhibits further mycorrhization, but also inhibits nodule formation. Conversely, already existing nodules systemically suppress both further nodulation and subsequent AMF colonization. These findings indicate that autoregulatory mechanisms induced by the establishment of one type of symbiosis influence another symbiotic association. This is reminiscent of certain supernodulating mutants of soybean and *Lotus japonicus*, which exhibited accelerated AMF colonization and increased formation of arbuscules compared to wild-type plants (Shrihari *et al*., 2000; Solaiman *et al*., 2000).

It remains an open question to what extent the mechanisms involved in autoregulation are identical for both symbioses. Induction of a systemic signal, the signal itself, perception of this signal in the other root part, and blocking mechanisms could be specific for a given symbiotic interaction. Our experiments with alfalfa plants suggest a link between the two symbioses regarding the systemic signal itself and its perception on the other side of the split-root system.

Recent results point towards a possible involvement of phytohormones in the rhizobial autoregulation. Apart from changes in the levels of other hormones, cytokinin levels are altered in roots of supernodulating mutants (Caba *et al*., 2000). No data are available yet on the role of phytohormones in the regulation of mycorrhization, however, hormone level changes have been reported in roots of plants colonized by AMF (e.g. cytokinin Allen *et al*., 1980; Shaul-Keinan *et al*., 2002).

Van Brussel *et al*. (2002) reported recently that application of Nod factors from *Rhizobium leguminosarum* bv. *viciae* to one side of a split-root system inhibited nodule formation of *Vicia* plants on the other side. Their observations indicate that Nod factors elicit an autoregulatory feedback-response. Our experiments show a similar effect of Nod factors in the interaction between alfalfa and *S. meliloti.* Moreover, it was found that application of Nod factors prevented AMF colonization on the other side of the split-root system. These findings indicate that Nod factors trigger a secondary signal involved in autoregulation of nodulation, which initiates a blockage of AMF colonization. In this context, it is worth mentioning that Nod factors may act as chito-oligosaccharide elicitors on legumes and non-legumes (Staehelin *et al*., 1994; Müller *et al*., 2000), thereby inducing plant defence reactions (Savouré *et al*., 1997; Xie *et al*., 1999). It is tempting to speculate that chito-oligosaccharides released from cell walls of AMF are stimuli triggering autoregulation of mycorrhization. The presence of AMF-derived signals acting on the plant even before appressoria formation has been demonstrated recently (Larose *et al*., 2002).

The observed effect of Nod factors on mycorrhization is not contradictory to the results from Xie *et al*. (1995, 1998), who reported that application of Nod factors to legume roots promoted AMF colonization. In their experiments, treatment with Nod factors was performed simultaneously with the mycorrhizal inoculation, moreover, the same roots were treated with Nod factors and inoculated with AMF, thus a local, but not a systemic effect was studied. Hence, Nod factors locally promote symbiosis and desensitize other parts of the roots via an autoregulatory feedback mechanism in both the rhizobial and mycorrhizal interactions.

Competition for carbohydrates within the root system is suggested to influence mycorrhizal colonization (Pearson *et al*., 1993). It is possible therefore that C-partitioning in alfalfa roots is involved in the autoregulation of symbiosis. Contrary to other AMF, however, *G. mosseae* strain BEG12 used in our experiment, does not induce any carbon sink strength in split-root systems (Lerat *et al*., 2003). In this work, it is shown that symbiotically active Nod factors from *S. meliloti* did not affect the allocation of ^{14}C between the two parts of an alfafa split-root system, although other Nod factors locally changed the carbohydrate composition in *Lablab purpureus* roots (Xie *et al*., 1998). Taking these observations together, they suggested that autoregulation of symbiosis cannot be explained by competition for carbohydrates.

Future work is required to test whether autoregulation of symbiosis is related to salicylic acid (SA) accumulation and defence responses resulting in systemic acquired resistance (SAR) to pathogen attack (Martinez-Abarca *et al*., 1998; Dong, 2001; Ramu *et al*., 2002).

There are some indications for the involvement of SA in the regulation of nodulation and mycorrhization. In transgenic NahG *Lotus japonicus* plants with reduced SA levels, an increased number of nodules is formed (McAlvin *et al*., 2001) and in NahG tobacco with reduced levels of SA and in transgenic CSA (constitutive SA biosynthesis) tobacco with enhanced SA levels it could be shown that the degree of root colonization by AMF is linked to the SA levels in the roots. Higher SA levels (in CSA plants) resulted in a reduced root colonization, whereas in roots with lower SA levels (in NahG plants) root colonization was increased (Herrera-Medina *et al*., 2003).

Several studies indicate that mycorrhizal roots are more resistant to pathogens (reviewed by Azcon-Aguilar and Barea, 1996) and that legumes inoculated with rhizobia are less damaged by soil-borne pathogens (Chakraborty and Purkayastha, 1984; Chakraborty and Chakraborty, 1989; Tu, 1978). Indeed, it seems plausible that symbiotic roots

develop systemic mechanisms to repulse colonization by pathogens, not discriminating between microsymbionts and soil-borne pathogens (Vierheilig and Piché, 2002). In this view, autoregulation would be a general strategy to resist pathogen attack, while reducing the costs for the already established symbiotic associations.

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