

## The *Rhizobium* sp. strain NGR234 systemically suppresses arbuscular mycorrhizal root colonization in a split-root system of barley (*Hordeum vulgare*)

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Nitrogen-fixing bacteria (rhizobia) form a nodule symbiosis with legumes, but also induce certain effects on non-host plants. Here, we used a split-root system of barley to examine whether inoculation with *Rhizobium* sp. strain NGR234 on one side of a split-root system systemically affects arbuscular mycorrhizal (AM) root colonization on the other side. Mutant strains of NGR234 deficient in Nod factor production (strain NGR $\Delta$ *nodABC*), perception of flavonoids (strain NGR $\Delta$ *nodD1*) and secretion of type 3 effector proteins (strain NGR $\Omega$ *rhcN*) were included in this study. Inoculation resulted in a systemic reduction of AM root colonization with all tested strains. However, the suppressive effect of strain NGR $\Omega$ *rhcN* was less pronounced. Moreover, levels of salicylic acid, an endogenous molecule related to plant defense, were increased in roots challenged with rhizobia. These data indicate that barley roots perceived NGR234 and that a systemic regulatory mechanism of AM root colonization was activated. The suppressive effect appears to be Nod factor independent, but enhanced by type 3 effector proteins of NGR234.

### Introduction

More than 80% of all land plants establish an arbuscular mycorrhizal (AM) symbiosis. In this interaction, arbuscular mycorrhizal fungi (AMF) colonize roots of host plants and promote plant growth through an improved nutrient supply, especially up-take of phosphorus (Smith and Read 1997). In legumes, symbiotic bacteria (rhizobia) induce the formation of root nodules, in which bacteroids fix atmospheric nitrogen. In response to flavonoids or other phenolic compounds in the rhizosphere, rhizobia secrete lipo-chitooligosaccharidic nodulation signals, so-called Nod factors (Perret et al. 2000). Other symbiotic determinants play a role in nodule formation of specific host legumes. *Rhizobium*

sp. strain NGR234, for example, secretes effector proteins (type 3 effectors secreted via the bacterial type 3 secretion system), which either promote or inhibit nodule formation in a host-specific manner (Dai et al. 2008, Kambara et al. 2009, Marie et al. 2003, Skorpil et al. 2005, Viprey et al. 1998).

A combined inoculation of legumes with both microsymbionts resulted in a higher root colonization by AMF and rhizobia in various studies (Chaturvedi and Singh 1986, Daft and El-Giahmi 1974, Pacovsky et al. 1986, Xie et al. 1995). A different picture was observed when one of the microsymbionts had colonized the root system of a legume plant prior to the other. Root colonization of a first symbiont resulted in the suppression of a second, later colonizing symbiont (Bethlenfalvay et al.

**Abbreviations** – AM, arbuscular mycorrhizal; AMF, arbuscular mycorrhizal fungi; FW, fresh weight; SA, salicylic acid.

1985). Recently, Catford et al. (2003, 2006) found that this antagonistic effect is systemically regulated. When one side of a split-root system of alfalfa plants was pre-inoculated with *Sinorhizobium meliloti*, subsequent AM root colonization on the other side of the split-root system was drastically reduced.

It has been reported that non-legumes also reacted to the presence of rhizobia in the rhizosphere (Plazinsky et al. 1985, Terouchi and Syono 1990). In rice and wheat, for example, rhizobia colonized the root surface and intercellular spaces between injured epidermal cells (Reddy et al. 1997, Webster et al. 1997). Furthermore, Nod factors can be perceived by non-legumes, such as tomato, as indicated by alkalization of the medium in cell cultures (Staehein et al. 1994). Interestingly, rhizobia share characteristics with plant growth promoting rhizobacteria that colonize the root surface. In the presence of rhizobia, growth of maize, lettuce, radish, rice and wheat was increased (Antoun et al. 1998, Chabot et al. 1996, Höflich et al. 1995, Webster et al. 1997). In addition to this plant growth promoting effect, rhizobia have also been associated with disease suppressive effects in legumes (Avis et al. 2008, Bardin et al. 2003, Chakraborty and Chakraborty 1989, Chakraborty and Purkayastha 1984, Elbadry et al. 2006, Huang et al. 2007, Siddiqui et al. 2007, Tu 1978). So far it is not known, however, whether rhizobia systemically affect mycorrhization in non-legumes. Here, we tested whether inoculation of barley plants with *Rhizobium* sp. strain NGR234 on one side of a split-root system systemically affects AM root colonization on the other side. Our data indicate that barley roots perceive NGR234 bacteria and activate a systemic regulatory mechanism that culminates in suppression of mycorrhization.

## Materials and methods

### Plant material and experimental set-up (split-root system)

Barley (*Hordeum vulgare* L. cv. Xanadu) seeds were surface sterilized by soaking in 0.75% sodium hypochlorite for 5 min, rinsed with tap water and germinated in vermiculite. Five-day-old seedlings were transferred to a steam-sterilized (20 min, 121°C) mixture of silicate sand, expanded clay and soil (1:1:1 v/v) into split-root systems consisting of compartment boxes (5 plants per compartment).

The split-root-experimental system consisted of two compartment units, each containing half of the barley-root system (for details, see Vierheilig et al. 2000). The two compartments were separated on the side joining

each other by an impermeable PVC membrane in order to prevent any flow of molecules, rhizobia or root and hyphal growth from one side to the other side. Thus, one side of the split-root system could be inoculated with rhizobia without inoculating the other side (Catford et al. 2003). The sides of each split-root compartment were equipped with a nylon screen (60 µm mesh), which allowed penetration of AM hyphae, but not of roots.

To inoculate half of the split-root system with *Glomus mosseae* (Nicolson and Gerdemann) Gerd and Trappe (BEG 12; European Bank for the *Glomales*), one side of a split-root compartment was joined to an inoculum compartment. The inoculum compartment contained beans (*Phaseolus vulgaris* L. cv. Sun Gold) colonized by *G. mosseae* and was equipped with a nylon screen (60 µm mesh) on one side. Thus, the AM hyphae could grow from the inoculum compartment through nylon screens to the split-root system, thereby rapidly colonizing the exposed half of the split-root system.

Experiments were performed in a growth chamber (day/night cycle: 16 h; 23°C/8 h; 19°C; relative humidity 50%). Plants were watered three times a week.

### Rhizobial and fungal inoculation

*Rhizobium* sp. NGR234 and mutant strains (NGRΔ*nodABC*, NGRΩ*nodD1*, NGRΩ*rhcN*; see Table 1) were cultured in TY broth (containing tryptone and yeast extract) or modified GMS medium (Staehein et al. 2006) supplemented with appropriate antibiotics on a rotary shaker (in 250 ml flasks; 150 r.p.m., 2 cm stroke length; at 28°C in the dark; final OD<sub>600</sub> ≈ 1). The bacterial cells were harvested by centrifugation in a sterile tube at 4500 g for 20 min. Thereafter, the supernatant was discarded and the bacterial pellet was re-suspended in sterilized 10 mM MgSO<sub>4</sub> to obtain a suspension of OD<sub>600</sub> = 0.2 (≈ 2 × 10<sup>8</sup> bacteria ml<sup>-1</sup>).

Two days after transferring barley plants into the split-root compartments (5 plants per split-root compartment), plants were inoculated on one half of the split-root system with 5 ml of the prepared rhizobial suspension. Control split-root systems were mock inoculated with 5 ml of MgSO<sub>4</sub> solution.

Two weeks after rhizobial inoculation, the second side of the split-root system was inoculated with AMF by joining the split-root compartment with an AMF inoculum compartment. Plants were harvested 3 weeks after AM inoculation.

The experiment was performed once with five replicates per treatment.

**Table 1.** *Rhizobium* sp. strain NGR234 and mutant strains used in this study. Rif<sup>R</sup>, resistance to rifampin; Sp<sup>R</sup>, resistance to spectinomycin.

Strains	Characteristics	References
<i>Rhizobium</i> sp. NGR234	Broad-host-range <i>Rhizobium</i> strain isolated from nodules of <i>Lablab purpureus</i> (Rif <sup>R</sup> )	Trinick et al. (1980), Stanley et al. (1988)
<i>Rhizobium</i> sp. NGR $\Delta$ nodABC	Mutant derivative of NGR234, NodABC <sup>-</sup> , no Nod factor synthesis, no nodule formation (Rif, Sp <sup>R</sup> )	Price et al. (1992)
<i>Rhizobium</i> sp. NGR $\Omega$ nodD1	NGR234 derivative containing an $\Omega$ cassette inserted to the <i>Bam</i> H1 site of <i>nodD1</i> , no upregulation of symbiotic NodD1-regulated genes in response to host flavonoids (Rif <sup>R</sup> , Sp <sup>R</sup> )	Relic et al. (1993)
<i>Rhizobium</i> sp. NGR $\Omega$ rhcN	NGR234 derivative containing an $\Omega$ cassette inserted to the <i>Eco</i> R1 site of <i>rhcN</i> , no functional type 3 secretion system (Rif <sup>R</sup> , Sp <sup>R</sup> )	Viprey et al. (1998)

### Determination of AM root colonization

At the time of harvest roots were carefully rinsed with water. Shoots and roots (from each side of the split-root system) were separated and their fresh weight (FW) determined. To quantify the degree of AM root colonization, roots were cleared (boiling in 10% KOH for 5 min) and then stained by boiling for 5 min in a 5% ink (Shaeffer; jetblack)/household vinegar (=5% acetic acid) solution (Vierheilig et al. 1998). Roots were destained in tap water, which was acidified with several drops of vinegar. Stained fungal structures in roots were observed with a stereo-microscope to evaluate the percentage of root colonization. The percentage of root colonization was determined according to a modified gridline intersect method of Newman (1966) by counting the mycorrhizal status of a minimum of 100 root sections when crossing gridlines in a petri dish.

### Extraction and quantification of salicylic acid

For the determination of salicylic acid (SA) levels, roots were rinsed with water, frozen ( $-80^{\circ}\text{C}$ ) and thereafter lyophilized. Free SA was extracted and quantified as described by Malamy et al. (1992). One gram of frozen root tissue was ground in 3 ml of 90% methanol and centrifuged at 6000 *g* for 15 min. The pellet was re-extracted with 3 ml of 100% methanol and centrifuged. Methanol extracts were combined, centrifuged for 10 min and dried at  $40^{\circ}\text{C}$  under vacuum. For each sample, the dried methanol extract was re-suspended in 5 ml of water at  $80^{\circ}\text{C}$ , and the solution was divided into two equal portions. To one portion (SAG), an equal volume of 0.2 M acetate buffer (pH 4.5) containing 2 U ml<sup>-1</sup>  $\beta$ -glucosidase (Sigma) was added, while to the other portion (free SA analysis) contained only buffer. Both portions were incubated at  $37^{\circ}\text{C}$  overnight. After digestion, samples were acidified with HCl to pH 1 and SA was extracted (and back extracted) with two volumes of cyclopentane/ethyl acetate/isopropanol (50:50:1). The

organic extract was dried under nitrogen, re-suspended in 50  $\mu\text{l}$  of 100% methanol and analyzed by HPLC.

Ten microliter of methanolic extract was injected into a C8 column (Varian) and phenolic compounds were separated with 30% (v/v) methanol in 1% acetic acid with a flow rate of 1.0 ml min<sup>-1</sup> at  $40^{\circ}\text{C}$ . SA was identified with a fluorescence detector set (Varian) at 310 and 405 nm (excitation and emission, respectively) and quantified with a Data Module, using authentic standards. As described previously for this method (Yalpani et al. 1993), recovery of SA ranged between 30 and 50%.

## Results

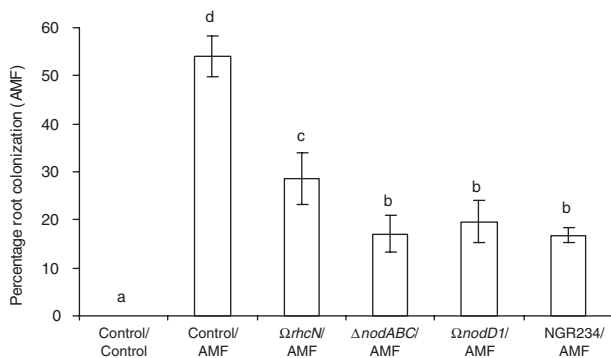
### Systemic effects of *Rhizobium* sp. strain NGR234 on mycorrhizal root colonization

To test the effect of *Rhizobium* sp. strain NGR234 on barley plants, roots were inoculated on one side of the split-root system with a suspension containing  $\approx 10^9$  bacteria. For comparison, control plants were left non-inoculated. Four weeks after inoculation, plants were harvested and plant growth was estimated by measuring the accumulated biomass of both sides of the split-root system as well as the shoot. As shown in Table 2, inoculation with NGR234 did not significantly affect growth of barley under the tested conditions.

In a similar split-root system experiment, the effect of prior rhizobial inoculation on AM root colonization was

**Table 2.** Inoculation of barley split roots with *Rhizobium* sp. NGR234 does not affect plant growth. Split-root systems were inoculated on the first side. Data indicate means  $\pm$  SD ( $n = 5$ ) at the time of harvest (4 weeks after inoculation).

Inoculum	Root FW on the first side of split-root system (g)	Root FW on the second side of split-root system (g)	Shoot FW (g)
<i>Rhizobium</i> sp. strain NGR234	0.34 $\pm$ 0.10	0.34 $\pm$ 0.11	1.05 $\pm$ 0.15
No bacteria	0.32 $\pm$ 0.13	0.33 $\pm$ 0.09	1.03 $\pm$ 0.22



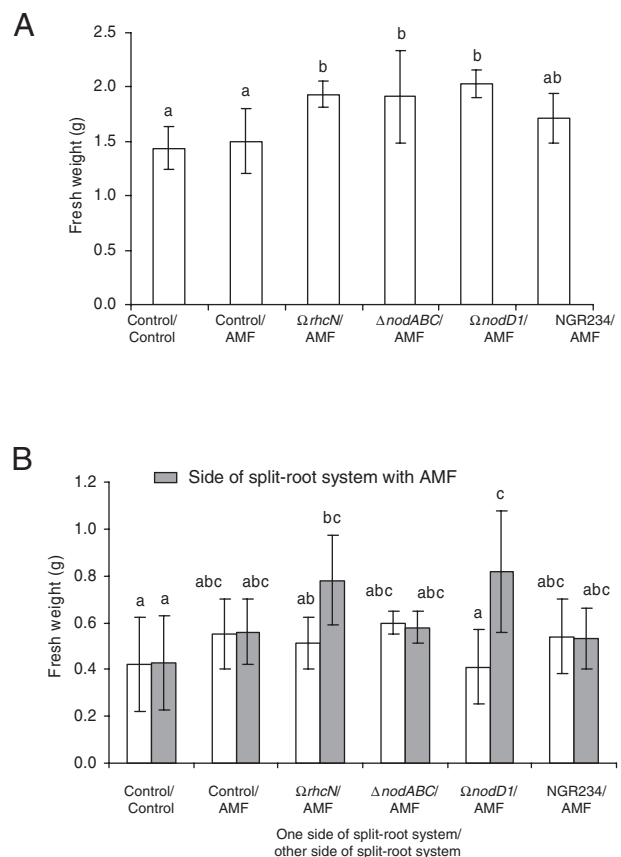
**Fig. 1.** Degree of AM root colonization in a split-root experiment with barley plants (5 plants per compartment;  $n = 5$ ). One side was pre-inoculated with *Rhizobium* sp. NGR234 or indicated mutants and the other side inoculated with the AMF *G. mosseae*. Control, no inoculation. Columns with the same letter are not significantly different according to the Tukey honestly significant difference (HSD)-test ( $P < 0.05$ ).

examined. Split roots were first inoculated with NGR234 on one side and 2 weeks later with the AMF *G. mosseae* on the other side (Fig. 1). At the time of harvest, highest levels of AM root colonization (more than 50%) occurred in control plants, which were not inoculated with bacteria. Inoculation with NGR234 significantly reduced AM root colonization (less than 20%), indicating that the bacterial inoculation systemically suppressed AM root colonization in other parts of the root system. Mutants of NGR234 deficient in Nod factor synthesis (strain  $\text{NGR}\Delta nodABC$ ) and perception of flavonoids (strain  $\text{NGR}\Omega nodD1$ ) also strongly suppressed establishment of the AM symbiosis. Interestingly, a significant lower suppressive effect was found for  $\text{NGR}\Omega rhcN$ , a mutant with a deficient type 3 secretion system (Fig. 1).

Compared to non-inoculated plants, inoculation with the tested strains and AMF resulted in promotion of plant growth. Plants with split-root systems that were inoculated with rhizobia on one side and AMF on the other side showed increased shoot biomass values (Fig. 2A). Inoculation with strains  $\text{NGR}\Omega rhcN$  and  $\text{NGR}\Omega nodD1$  resulted in a significant increase of the root biomass colonized by AMF in the other half of the split-root system. However, this effect was not seen for NGR234 and  $\text{NGR}\Delta nodABC$  mutant (Fig. 2B).

### Accumulation of SA in response to inoculation with rhizobia

To characterize perception of NGR234 bacteria by barley roots, in more detail, effects of rhizobial inoculation on levels of SA were examined. Lowest accumulation of free SA was detected in non-inoculated roots of control plants. Inoculation with NGR234 and all tested mutant

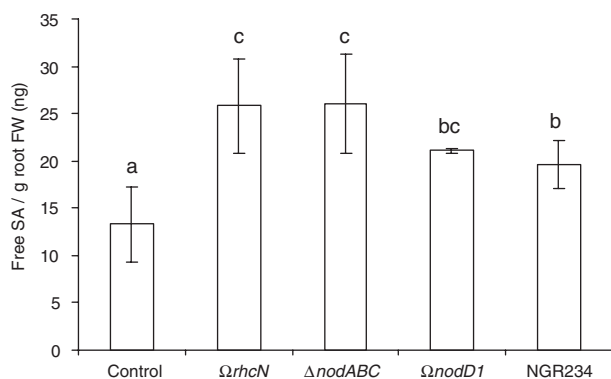


**Fig. 2.** Shoot (A) and root (B) biomass of barley plants in a split-root experiment with one side pre-inoculated with *Rhizobium* sp. NGR234 or indicated mutants and the other side inoculated with AMF *G. mosseae* (5 plants per compartment;  $n = 5$ ). Control, no inoculation. Columns with the same letter are not significantly different according to the Tukey HSD-test ( $P < 0.05$ ).

derivatives significantly enhanced the levels of free SA (Fig. 3).

### Discussion

It has been reported that non-legumes are able to perceive the presence of rhizobia or Nod factors (Antoun et al. 1998, Chabot et al. 1996, Höflich et al. 1995, Plazinsky et al. 1985, Staehelin et al. 1994, Terouchi and Syono 1990, Webster et al. 1997). Other studies indicated plant growth promoting effects induced by various rhizobia, particularly on cereal crop plants such as maize, rice and wheat (Chabot et al. 1996, Höflich et al. 1995). Rhizobia also have been reported to be closely associated with roots of wheat and rice (Reddy et al. 1997, Webster et al. 1997). In this study, we used *Rhizobium* sp. NGR234, a broad-host-range strain with the remarkable ability to nodulate more than 110



**Fig. 3.** Concentration of free SA in barley roots inoculated with *Rhizobium* sp. NGR234 or indicated mutants (in the absence of AMF). Roots from 5 plants ( $n = 5$ ) were harvested 14 days after inoculation. Control, no inoculation. Columns with the same letter are not significantly different according to the Tukey HSD-test ( $P < 0.05$ ).

genera of legumes as well as the non-legume *Parasponia andersonii* (Pueppke and Broughton 1999). Our data provide evidence that barley roots are able to perceive strain NGR234 based on the findings that rhizobial inoculation affected AM root colonization, plant growth of mycorrhizal plants and accumulation of SA in root tissue.

In the experiments of this study, prior inoculation of one side of a split-root system with NGR234 resulted in a reduction of AM root colonization on the other side of the split-root system, indicating a systemic suppression mechanism activated by inoculation with NGR234. This result is reminiscent to data obtained from the legume alfalfa; when the first side of a split-root system was inoculated with *S. meliloti*, AM root colonization on the second side was drastically reduced (Catford et al. 2003, 2006). Hence, systemic suppression of AM root colonization caused by rhizobial inoculation seems to be a general phenomenon in legumes and non-legumes. In alfalfa plants, systemic suppression of AM root colonization was also observed when purified Nod factors were applied to the first side of the split-root system (Catford et al. 2003). In the present study with barley roots, however, a mutant deficient in Nod factor synthesis (strain NGRΔ*nodABC*) induced systemic suppression of AM root colonization, indicating that Nod factors are not required for the suppression mechanism. These differences suggest that legumes and non-legumes regulate systemic suppression of AM root colonization in a different way.

Compared to NGR234, strain NGRΩ*rhcN* exhibited a weaker effect on suppression of AM root colonization, and also promoted root growth in the mycorrhizal part of the split-root system. NGRΩ*rhcN* lacks a functional type

3 secretion system (Viprey et al. 1998) and consequently cannot secrete any type 3 effector proteins. Type 3 effectors of NGR234 are host-specific determinants of symbiosis, which can either promote or inhibit nodule formation of host legumes (Dai et al. 2008, Kambara et al. 2009, Marie et al. 2003, Skorpil et al. 2005, Viprey et al. 1998). In interactions between plants and bacterial phytopathogens, type 3 effectors are delivered into host cells, where they function as important virulence or avirulence factors (Büttner and Bonas 2006). The data of this study point to a role of type 3 effectors in the interaction between NGR234 and cereal non-host plants. Future work is required to test the effects of other rhizobial strains. Moreover, it would be interesting to investigate whether specific type 3 effectors affect rhizobial colonization of cereals and whether resistance proteins of barley are involved in recognition of rhizobial type 3 effectors.

The mutant NGRΩ*nodD1* was included in this study, as host flavonoids activate expression of many symbiotic genes, including those required for Nod factor synthesis and formation of a functional type 3 secretion system. Rhizobial NodD proteins are key components in perception of flavonoids. The NodD1 protein of NGR234 responded not only to various flavonoids, but also to the simple phenolic compounds, vanillin and isovanillin, from wheat seedling extracts (Le Strange et al. 1990). Expression of flavonoid-inducible genes is dramatically reduced in the NGRΩ*nodD1* mutant (Kobayashi et al. 2004). In the present experiment with barley plants, inoculation with the parent strain NGR234 and NGRΩ*nodD1* differed with respect to root biomass of the second half of the root system colonized by AMF, suggesting a role for NodD1-dependent genes of NGR234 in this process. This result is consistent with the observed difference between NGR234 and NGRΩ*rhcN* in our study, as activation of *tts1*, a transcriptional regulator of type 3 secretion genes, depended on NodD1 and flavonoids (Kobayashi et al. 2004, Wassem et al. 2008). Future experiments are required to study the expression of flavonoid-inducible genes of NGR234 in the rhizosphere of barley and to test whether exuded flavonoids or phenolic compounds modulate the ability of NGR234 to colonize the root surface.

SA is not only an endogenous molecule involved in plant defense (Klessig and Malamy 1994), but also seems to play role in the nodule symbiosis of legumes (Stacey et al. 2006). There is also some information available on the involvement of SA in the regulation of root colonization by AMF (Blilou et al. 1999, Ludwig-Müller et al. 2002, Medina et al. 2003), indicating that enhanced SA levels in plant tissues are correlated with reduced AM root colonization. In the present study,

rhizobial inoculation of barley roots increased the levels of free SA with all tested strains, suggesting induction of a defense reaction in response to a potential pathogen. Future experiments with non-legumes are required to search for rhizobial elicitor molecules involved in recognition of rhizobia and to study the role of SA and defense reactions during establishment of symbiosis with AMF.

To summarize, the data of this work clearly show a systemic suppressive effect of *Rhizobium* sp. strain NGR234 on AM root colonization in a non-legume. The capacity of NGR234 to suppress AMF did not depend on Nod factors, but was reduced in NGR $\Omega$ *rhcN*, a mutant with a deficient type 3 secretion system. Moreover, SA levels of barley roots were accumulated upon rhizobial inoculation. Future work is required to test a possible involvement of SA in the observed systemic effect on AM colonization.

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