



Short communication

First indications for the involvement of strigolactones on nodule formation in alfalfa (*Medicago sativa*)

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ABSTRACT

Strigolactones have recently been suggested to be phytohormones that are present in all plants. Strigolactones are released by roots into the rhizosphere, stimulating the seed germination of parasitic plants such as *Striga* spp. and *Orobanch* spp. and play a crucial role in the interaction between plants and symbiotic arbuscular mycorrhizal fungi.

By applying different concentrations of the synthetic strigolactone analogue GR24 to alfalfa (*Medicago sativa*) inoculated with *Sinorhizobium meliloti* we could show that in alfalfa nodulation is positively affected by the presence of the strigolactone analogue GR24. Moreover, we could show that this increased nodulation cannot be linked with a stimulatory effect of GR24 on the growth or the expression of *nod* genes of *S. meliloti*.

Putative mechanisms operating in the plant in response to the addition of GR24 and leading to increased nodule formation by rhizobia are discussed.

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Strigolactones have been identified as phytohormones involved in the regulation of shoot branching in plants and thus have been suggested to be ubiquitous in the plant kingdom (Gómez-Roldán et al., 2008; Umehara et al., 2008). Since long, strigolactones – that are released by roots into the rhizosphere – have been known as seed germination stimulants for parasitic plants such as *Striga* spp. and *Orobanch* spp. (Bouwmeester et al., 2007; López-Ráez et al., 2008a) and recently, their role as key signalling compounds in the interaction between plants and soil-borne symbiotic arbuscular mycorrhizal fungi has been suggested (Akiyama et al., 2005; Besserer et al., 2006).

Seed germination stimulants – including strigolactones – are exuded, although at varying levels, by the roots of all plants investigated so far (Berner and Williams, 1998; Fernández-Aparicio et al., 2009; Lenzemo et al., 2009; Steinkellner et al., 2007; Yoneyama et al., 2008), and thus a more general signalling role of these compounds in plant microbe interactions in the soil has been suggested (García-Garrido et al., 2009).

Scarce data are available on strigolactones and other fungi apart from AMF (García-Garrido et al., 2009; Martínez et al., 2001; Sabagh, 2008; Steinkellner et al., 2007), however, nothing is known yet whether strigolactones affect the *Rhizobium*-legume symbiosis,

characterized by the formation of nitrogen-fixing nodules on roots of legume plants (for a review see Gibson et al., 2008; Oldroyd and Downie, 2008).

There are some indications that strigolactones are somehow involved in the *Rhizobium*-legume interaction. In several studies with pea plants it has been reported that exudates from non-nodulated plants exhibit a higher seed germination activity on seeds of *Orobanch* than exudates from nodulated plants, indicating that the levels of seed germination stimulants – strigolactones – are reduced in nodulated plants (Mabrouk et al., 2007a,b,c), however, no data are available yet how strigolactones can affect nodulation. To analyze the role of strigolactones in the establishment of the *Rhizobium*-legume symbiosis, we assessed the formation of nodules on alfalfa (*Medicago sativa*) in presence of different concentrations of the synthetic strigolactone analogue GR24.

1. Bacterial strains and experimental conditions

Sinorhizobium meliloti strain 1021 (Meade and Signer, 1977) was grown routinely at 30 °C in TY complex medium (Beringer, 1974). To investigate the effect of GR24 (kindly provided by Prof. B. Zwanenburg; Stichting Chemiefonds Paddepoel; Malden/The Netherlands) on *S. meliloti* growth, cells were incubated in defined minimal medium (MM) (Robertsen et al., 1981) containing different concentrations (10^{-7} , 10^{-5} , and 10^{-3} M) of GR24. Growth was

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determined regularly with a spectrophotometer measuring the absorbance at 600 nm.

S. meliloti 1021 containing plasmid pRmM57 (*nodC::lacZ* fusion) (Mulligan and Long, 1985) was used to test the expression of the *nodC* gene and the same strain containing plasmid pGD499 (*npt::lacZ* fusion) (Ditta et al., 1985) was used to test the expression of the constitutive kanamycin resistance gene as a positive control.

To prepare GR24, 1 mg of the compound was dissolved in 33 μ l acetone to obtain a solution 10^{-1} M which was serially diluted in water to obtain the desired concentrations of GR24. The control contained only the solvent acetone but no GR24.

2. Measurement of β -galactosidase activity (*nod* gene expression)

S. meliloti cells containing *lacZ* fusions were grown in liquid MM containing tetracycline to ensure plasmid maintenance. Bacteria were grown in liquid cultures overnight at 30 °C to early logarithmic phase (OD₆₀₀ of 0.1–0.3) in the presence or absence of 5 μ M luteolin and different concentrations (10^{-7} , 10^{-5} , and 10^{-3} M) of GR24. Samples of 100 μ l of the bacterial culture were taken and assayed for β -galactosidase activity by the SDS-chloroform method described by Miller (1972).

3. Nodulation tests

Alfalfa (*M. sativa* L. cv. Aragon) seeds were sterilized and germinated as described by Olivares et al. (1980). One day-old seedlings were placed on sterile filter paper strips in glass tubes covered with an aluminium foil to keep roots in the dark (20 by 200 mm; 1 seedling per tube) containing 10 ml of nitrogen-free mineral solution (Rigaud and Puppo, 1975). Plants were grown under controlled conditions of light and temperature (16 h of light at 24 °C and 8 h of dark at 16 °C). For each treatment, twenty plants (ten days old) were inoculated with 1 ml of a suspension prepared in sterile water containing approximately 5×10^6 colony forming units (cfu) of *S. meliloti* 1021 per ml, and the appropriate amount of either GR24 or acetone as control. In addition a set of plants was grown in absence of *Rhizobium*.

Previously, *S. meliloti* 1021 cells were grown in liquid TY medium (Beringer, 1974) at 28 °C up to an OD₆₀₀ of 0.5 and then diluted accordingly. The following GR24 concentrations (10^{-5} , 10^{-7} , 10^{-8} and 10^{-9} M) were tested. After inoculation, the number of nodules per plant was recorded daily.

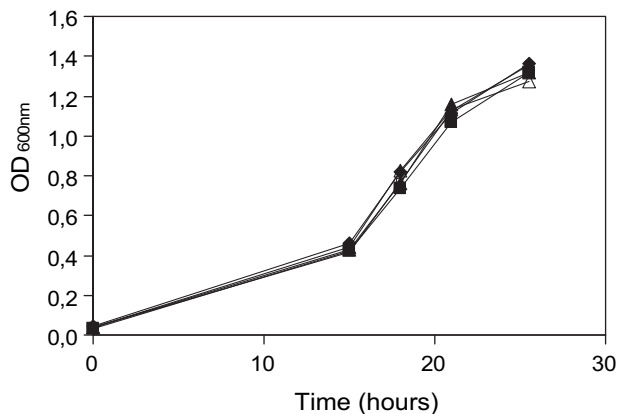


Fig. 1. Effect of different concentrations of GR24 on the growth of *S. meliloti* 1021. Growth of 1021 was tested in minimal medium (MM) with 0 M (diamonds), 10^{-5} M acetone (open triangles), 10^{-3} M GR24 (closed triangles), 10^{-5} M GR24 (open squares), and 10^{-7} M GR24 (closed squares).

Table 1

Effect of GR24 (10^{-7} M) on β -galactosidase activity of the *npt::lacZ* and *nodC::lacZ* fusions measured in the absence or in the presence of luteolin (5 μ M). Mean values and standard errors (95% confidence) were calculated from three independent experiments (MM = minimal medium).

Growth conditions	β -galactosidase activity (Miller U)	
	pGD499 (<i>npt::lacZ</i>)	pRmM57 (<i>nodC::lacZ</i>)
MM	789 \pm 105	84 \pm 7
MM + luteolin	840 \pm 66	391 \pm 18
MM + luteolin + GR24	832 \pm 49	329 \pm 13
MM + GR24	749 \pm 68	83 \pm 7

When looking at the effect of GR24 (10^{-3} , 10^{-5} and 10^{-7} M) on bacterial growth (Fig. 1) and *nod* gene expression (Table 1) we found that both parameters were not affected by GR24. For the *nod* gene expression data of the 10^{-7} treatment are given (Table 1). Similar results were obtained with the other two concentrations.

In the nodulation experiment no nodules were detected in the control plants without rhizobial inoculation. When looking at the effect of GR24 on nodulation we observed a clear stimulation of nodulation in the presence of 10^{-7} and 10^{-8} M of GR24 (Fig. 2), whereas 10^{-5} and 10^{-9} M of GR24 showed no effect on nodulation. The positive effect of GR24 on nodulation was similar after 14 d (Fig. 2) and 30 d (data not shown) after inoculation.

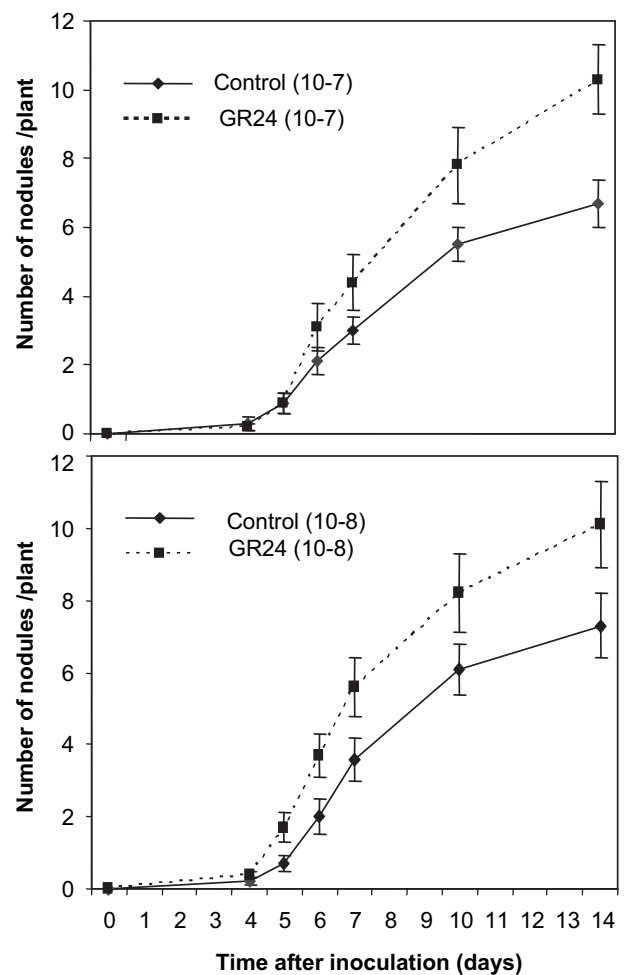


Fig. 2. Timecourse of nodulation in *Medicago sativa* inoculated with *Sinorhizobium meliloti* after application of different GR24 solutions and the corresponding control solutions. Strigolactone and control treatment were applied at the time of inoculation. Data represent mean number of nodules per plant ($n = 20$). Error bars represent standard errors at 95% confidence interval.

In both, control plants and plants treated with GR24, pink nitrogen-fixing nodules were formed. In none of the treatments differences of the plant growth (root or shoot) could be observed.

In our experiment the two concentrations 10^{-7} and 10^{-8} M of the synthetic strigolactone analogue GR24 showed a clear positive effect on nodulation. Interestingly these GR24 concentrations are known also to stimulate AM hyphal branching (Besserer et al., 2006; López-Ráez et al., 2008b), thus, showing their biological relevance. To check whether the greater nodule formation efficiency shown by GR24 correlates with an altered bacterial growth rate or *nod* gene expression, two of the most important rhizobial parameters influencing nodulation, we tested its effect on bacterial growth and induction of a *nodC::lacZ* fusion by luteolin. As none of the bacterial parameters was affected by the presence of the strigolactone analogue GR24 we conclude that the positive effect of the strigolactone analogue GR24 on nodulation is not due to an effect on the bacterial side.

This would mean that the strigolactone analogue acts on the plant side. Probably, when plants are treated with GR24, the biosynthesis and the metabolism of the strigolactones of alfalfa is affected, thus, resulting in an enhanced nodulation. Moreover, the application of GR24 might alter the levels of other phytohormones (auxin and cytokinin) with crucial roles in nodule organogenesis, as it has been recently reported that strigolactone levels affect the levels of these phytohormones (Arite et al., 2007; Hayward et al., 2009). Further tests are presently under way to elucidate the mechanism/s involved.

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