Bio-protective effect of a root-nodulating *Rhizobium etli* strain in common bean (*Phaseolus vulgaris*) against *Meloidogyne incognita* and *Radopholus similis* in an *in vitro* autotrophic tripartite culture system

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Summary – The bio-protective effect of a root-nodulating strain (CNP4F 512) of the nitrogen-fixing rhizobium, *Rhizobium etli*, against both a sedentary (*Meloidogyne incognita*) and a migratory (*Radopholus similis*) endoparasitic nematode in common bean (*Phaseolus vulgaris*) was examined using an *in vitro* autotrophic tripartite culture system. Two *in vitro* assays were carried out with each of the nematode species. Each assay consisted of two treatments: the plants were either inoculated with the rhizobial strain or remained non-inoculated (control plants). To examine the effect of either pre- or simultaneous inoculation of the rhizobial strain on the reproduction of *M. incognita* and *R. similis*, one assay was carried out in which the nematodes were inoculated 3 weeks after rhizobial inoculation while another assay was carried out in which the nematodes were inoculated simultaneously with the rhizobial strain. Both pre-inoculation and simultaneous inoculation with *R. etli* CNP4F 512 significantly suppressed the reproduction of both *M. incognita* and *R. similis*.

Keywords – autotrophic model system, biocontrol, burrowing nematode, nodulation, plant growth-promoting rhizobacteria, root-knot nematode.

Common bean (*Phaseolus vulgaris* L.) is the most widely cultivated food legume throughout the world, providing a primary source of dietary protein, fibre, starch and minerals affordable by the poor (Broughton *et al.*, 2003; Martinez-Romero, 2003). It is considered an ‘all-around’ crop that can be grown either as a rotation crop, intercrop or green manure crop in a wide range of agro-

ecological systems (Broughton *et al.*, 2003). As with most other species of the legume family (Fabaceae), common bean can form a symbiosis with nitrogen-fixing soil bacteria (collectively known as rhizobia) within a specialised structure, the root nodule (van Rhijn & Vanderleyden, 1995; Martinez-Romero, 2003; Soares *et al.*, 2016). Rhizobia can take up gaseous dinitrogen (N₂) from the air

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and ‘fix’ it into ammonia that can subsequently be assimilated into amino acids by the bacterium or the plant. In return, the plant provides the rhizobia with a carbon source in the form of dicarboxylic acids (van Rhijn & Vanderleyden, 1995). Although common bean has good potential for N₂ fixation, it was reported to have the lowest N₂ fixation rate among the most widely grown grain legumes (Martinez-Romero, 2003). Most of the rhizobia originally isolated from tropical legumes from humid or sub-humid tropical forests from the Amazon and the south-east of Brazil (the centre of origin of common bean) were able to induce nodules or nodular structures on common bean, but only a minority of these nodules were also nitrogen-fixing, indicating a high specificity for effective N₂ fixation (Michiels *et al.*, 1998). A nitrogen-fixing plant produces leghaemoglobin, a protein related to human haemoglobin, for providing oxygen to the nodules, giving functional nodules a pink colour (Soares *et al.*, 2016). *Rhizobium etli* is the predominant species found in the centres of origin of common bean in Central and South America (Martínez-Romero, 2003).

Everywhere throughout the tropics and subtropics common bean is attacked by a diversity of plant-parasitic nematodes, among which the root-knot nematodes (*Meloidogyne* spp.) are the most important. The most common root-knot nematode species infecting common bean are *Meloidogyne incognita*, *M. javanica* and *M. arenaria* (Sikora *et al.*, 2018). Root-knot nematodes are sedentary endoparasites. Also, root-lesion nematodes (*Pratylenchus* spp.) have been reported to infect common bean causing extensive root necrosis and yield loss (Sikora *et al.*, 2018). The most typical symptom caused by root-knot nematodes are galls (knots), and by root-lesion nematodes root lesions, purplish-black necrotic areas that usually extend throughout the cortex (Jones *et al.*, 2013).

Rhizobia belong to the group of plant growth-promoting rhizobacteria (PGPR) that are able to enhance the tolerance of their hosts to both abiotic and biotic stresses (Verma *et al.*, 2019). Research on the effect of root nodulation by rhizobia on plant-parasitic nematodes and *vice versa* has mainly dealt with the interaction between soybean and *Heterodera glycines*, the soybean cyst nematode, and between a variety of leguminous crops and the most common root-knot nematode species, especially *M. incognita*. In most instances, root nodulation by rhizobia suppressed root galling and reproduction of *M. incognita* and improved plant growth (Huang, 1987; Fazal *et al.*, 1992; Khan *et al.*, 2018), whilst infection by *M. incognita* reduced either the number of nodules, their functionality or

induced premature senescence of the nodules (see, for example, Baldwin *et al.*, 1979; Huang, 1987; Khan *et al.*, 2002, 2018; Neeraj & Singh, 2019). Similarly, infection by *Pratylenchus penetrans* affected the number, weight and functionality of nodules (Elhady *et al.*, 2020). Information on the effect of root nodulation by rhizobia on migratory endoparasitic nematodes is scarce. Recently, Van der Veken *et al.* (unpubl.) found that simultaneous inoculation of soybean with *R. etli* CNPAF 512 and *Radopholus similis*, a root-lesion nematode, significantly suppressed reproduction of this migratory endoparasite.

The objective of our study was to evaluate the bio-protective effect of the root-nodulating strain *R. etli* CNPAF 512 against both a sedentary (*M. incognita*) and a migratory (*R. similis*) endoparasitic nematode in common bean. The experiment was carried out using an *in vitro* autotrophic tripartite culture system.

Materials and methods

PLANT MATERIAL

Common bean ‘BAT 477’ seeds were obtained from the Centre of Microbial and Plant Genetics (CMPG), University of Leuven, Belgium. This cultivar was selected because its roots develop well *in vitro* and it is susceptible to both *M. incognita* and *R. similis*. The seeds were placed in a Miracloth pouch in a sterilised Erlenmeyer flask and surface-sterilised by submersion in 97% ethanol for 1 min and 15% NaOCl for 12 min. The Erlenmeyer flask was shaken constantly to assure close contact between the seeds and the disinfectants. The seeds were then rinsed ten times with sterile demineralised water (dH₂O) for 1 min and soaked in dH₂O for 1 h. Subsequently, the seeds were removed from the Miracloth pouch with sterilised tweezers and 4-5 sterilised seeds transferred to sterilised 9-cm diam. Petri dishes containing a sterilised water agar medium for germination. The Petri dishes were sealed with parafilm and placed in an incubator at 25°C with a 12:12 light:dark photoperiod.

BACTERIAL INOCULUM

The *R. etli* CNPAF 512 strain was also obtained from CMPG. This bacterial strain was selected because of its good compatibility with common bean ‘BAT 477’ (Michiels *et al.*, 1998). The bacterial strain was first cultured for 2-3 days at 28°C on a solid tryptone-yeast (TY) medium containing 5 g tryptone, 3 g yeast extract

Fig. 1. A: *In vitro* autotrophic tripartite culture system of common bean; B: Nodulation of roots of common bean inoculated with *Rhizobium etli* CNPAF 512; C: Egg mass of *Meloidogyne incognita* after Phloxine B staining.

with 15 g plant agar l⁻¹ (Bittinger & Handelsman, 2000) followed by 1 day on a liquid TY medium at 28°C and 200 rpm. Then 1% of a 10 mM CaCl₂·H₂O solution was added to promote growth of the bacterial cell wall. The optical density (OD) of the bacterial suspension was determined by measuring its absorbance at 600 nm wavelength using a spectrophotometer. At the time of inoculation, the bacterial suspension was diluted in a 10 mM MgSO₄ solution to obtain a bacterial inoculum containing about 10⁶ colony forming units (CFU) ml⁻¹.

NEMATODE INOCULUM

The *M. incognita* population used in the trials was originally collected from a banana field in Malaysia and maintained on susceptible tomato 'Marmande' plants in soil in pots in a glasshouse. The inoculum consisted of infective second-stage juveniles (J2) that were extracted from infected tomato roots by a modified Baermann technique. Under sterile conditions in a laminar flow chamber and using a stereomicroscope, nematode egg masses protruding from infected tomato roots were removed with a scalpel and transferred to a 20-mm-diam. microsieve (70-μm diam. mesh), which was placed in the centre of a watch glass containing sterile dH₂O. The watch glass was then placed in a Petri dish which was subsequently sealed with parafilm and placed in an incubator at 25°C in the dark. Six days later, the majority of J2 had hatched and were collected by rinsing the watch glass with sterile dH₂O.

The *R. similis* population used in the trials was originally collected from a banana field in Uganda and maintained on a monoxenic alfalfa callus culture grown on a modified White's medium in Petri dishes at 27°C in the dark (Elsen *et al.*, 2001). The inoculum consisted of approximately 60 females, which were collected under ster-

ile conditions in a laminar flow chamber by rinsing the callus medium with sterile dH₂O.

EXPERIMENTAL SET-UP

To examine the bio-protective effect of root nodulation on *M. incognita* and *R. similis* on common bean, an *in vitro* autotrophic tripartite culture system was developed based on an autotrophic culture system for the *in vitro* mycorrhization of potato plantlets (Voets *et al.*, 2005; Fig. 1). This system consisted of sterilised 14.5-cm diam. closed and sealed Petri dishes containing a pH-adjusted (pH 6.2) sugar- and nitrogen-free Snoeck agar medium (Snoeck *et al.*, 2003). The root system of a sterilised 5-day-old common bean 'BAT 477' seedling was gently pushed through an opening (made in the side of each of the Petri dishes with a heated cork borer) and into the medium until the tap rootlet reached the centre of the Petri dish. After insertion of the root system, the opening in the side of the Petri dish was sealed with silicon grease. In this way, an even growth of the seedlings' roots over the medium inside the Petri dishes could be obtained, while the shoots were growing outside the Petri dishes to allow normal photosynthesis.

Two *in vitro* assays were carried out with each of the nematode species. Each assay consisted of two treatments: the plants were either inoculated with the rhizobial strain (RHIZ⁺) or remained non-inoculated (RHIZ⁻; control plants). To examine the effect of either pre- or simultaneous inoculation of the rhizobial strain on the reproduction of *M. incognita* and *R. similis*, one assay was carried out in which the nematodes were inoculated 3 weeks after rhizobial inoculation while another assay was carried out in which the nematodes were inoculated simultaneously with the rhizobial strain. Petri dishes were arranged in a randomised block design. Each treatment was replicated

either six or eight times. The simultaneous inoculation of *R. etli* CNPAF 512 and *M. incognita* assay was repeated.

Rhizobial inoculation (RHIZ⁺) was done by pipetting 1 ml of the *R. etli* CNPAF 512 inoculum suspension (containing $\pm 10^6$ CFU) over the 5-day-old tap rootlets, allowing any excess of the suspension to drip onto the medium. Control plants (RHIZ⁻) only received 1 ml of the 10 mM MgSO₄ solution. For the pre-inoculation treatment, the nematodes were inoculated 3 weeks after inoculation with *R. etli* CNPAF 512, when nodules had already formed. A 1 ml aqueous suspension containing either ca 60 J2 of *M. incognita* or ca 60 females of *R. similis* was pipetted on the medium near the root tips of the plantlets (both RHIZ⁺ and RHIZ⁻ plants). For the simultaneous inoculation treatment, the nematodes were inoculated as described above at the same time as the rhizobial inoculation (both RHIZ⁺ and RHIZ⁻ plants). After inoculation, the Petri dishes were sealed with parafilm and the opening in the side of the Petri dish around the stems of the seedling sealed with silicon grease. Petri dishes were stacked randomly with the shoots facing the same direction, wrapped with aluminum foil to mimic underground conditions for optimal root growth and placed in a growth chamber at 25°C, 70% RH and a 12:12 light:dark photoperiod.

ASSESSMENT OF ROOT GROWTH, RHIZOBIAL COLONISATION AND NEMATODE REPRODUCTION

Eight weeks after nematode inoculation, the Petri dishes were opened, the root systems gently removed from the medium and rinsed with tap water. The length of the tap root was measured. Rhizobial colonisation was assessed by counting the number of nodules that had developed on the whole root system using a stereomicroscope (Fig. 1B) while the inside colour of the nodules was observed for the presence of leghaemoglobin. For *M. incognita*, egg-laying females (ELF) were stained red (Fig. 1C) by soaking the rinsed root systems in a Phloxine B solution (0.15 g l⁻¹) for 15 min (Holbrook *et al.*, 1983), and ELF and egg masses counted using a stereomicroscope. The J2 and males were extracted from the medium using a Baermann tray (Hooper *et al.*, 2005) and counted after 48 h of extraction in two 2 ml subsamples of a 50 ml suspension using a light microscope. For *R. similis*, root necrosis was first scored on a 0 to 7 scale: 0 = absence of lesions; 1 = sporadic lesions and traces of infections in <5% of the roots; 2 = larger coalesced lesions in 5 to up to 10% of the roots; 3 = necrotic root parts present in 10 to up to 25% of the roots; 4 = 25% of roots

necrotic and lesions visible in the other roots; 5 = between 25 and up to 50% of roots necrotic and lesions visible in the other roots; 6 = between 50 and up to 75% of roots necrotic and lesions visible in the other roots; 7 = >75% of roots necrotic. Subsequently, juveniles and adults were extracted from the roots by the maceration-sieving technique (Hooper *et al.*, 2005) and from the medium as described for *M. incognita* above. Number of eggs, juveniles, females and males were counted in two 2 ml subsamples of a 50 ml suspension using a light microscope.

STATISTICAL ANALYSIS

Data were analysed separately for each assay. Nematode data were log (x + 1) transformed prior to statistical analysis. One-way ANOVA and post-hoc Tukey equal HSD test were performed using Statistica 7.1 software after verifying the ANOVA assumptions (Anonymous, 2007). The categorical root necrosis data were analysed using the Kruskal-Wallis test.

Results

In all assays, the common bean plantlets developed a good root system (Fig. 1A, B) with root lengths averaging 20–25 cm (assays with *M. incognita*). Rhizobial inoculation was also successful in all assays. On the plants of the pre-inoculation assays infected with *M. incognita*, on average 43 and 27 nodules were counted in trials 1 and 2, respectively, while on the plants of the simultaneous inoculation assay with *M. incognita*, on average 87 nodules were counted (Fig. 2A). On the plants of the pre-inoculation and simultaneous inoculation assays infected with *R. similis*, on average 45 and 71 nodules, respectively, were counted (Fig. 2B). Active nitrogen fixation by the rhizobia was observed visually by the presence of nodules with a pink centre, indicating the presence of leghaemoglobin.

Pre-inoculation with *R. etli* CNPAF 512 significantly ($P \leq 0.05$) reduced the number of *M. incognita* ELF, egg masses in the roots (by 40 and 50%) and number of J2 in the medium (by 57 and 59%) but not the number of males in both trials 1 and 2, respectively (Table 1). A similar result was obtained in the simultaneous inoculation assay, but in this assay the number of males was also significantly ($P \leq 0.05$) reduced (by $\pm 40\%$). In the simultaneous inoculation assay, a much higher number of males of *M. incognita* was observed compared with the pre-inoculation assay in both non-inoculated plants and plants

Fig. 2. Mean number of nodules per root system that had developed on common bean 8 weeks after inoculation with *Meloidogyne incognita* (A) or *Radopholus similis* (B). Nematode inoculation was done either 3 weeks after inoculation with *Rhizobium etli* CNPAF 512 (pre-nodulation) or simultaneously with inoculation of the rhizobial strain. Bars indicate standard errors of the means.

Table 1. Effect of pre-inoculation with *Rhizobium etli* followed 3 weeks later by inoculation with ca 60 second-stage juveniles (J2) of *Meloidogyne incognita* (pre-inoculation assay) and simultaneous inoculation with both *R. etli* and ca 60 J2 of *M. incognita* (simultaneous inoculation assay) on the reproduction of *M. incognita* and root length of common bean plants, grown in an *in vitro* autotrophic tripartite culturing system, at 8 weeks after nematode inoculation.

Treatment	n	Number				Root length (cm)
		J2	Egg-laying females (ELF)	Egg masses	Males	
Pre-inoculation with <i>R. etli</i> followed by inoculation with <i>M. incognita</i>						
Trial 1						
RHIZ [−]	8	228	10	8	8	20.6
RHIZ ⁺	8	98	6	3	14	23.3
		**	***	***	ns	ns
Trial 2						
RHIZ [−]	8	355	10	8	16	20.0
RHIZ ⁺	8	147	5	3	23	22.7
		***	***	***	ns	ns
Simultaneous inoculation of <i>R. etli</i> and <i>M. incognita</i>						
RHIZ [−]	6	216	9	8	206	25.0
RHIZ ⁺	6	73	5	3	125	23.1
		***	***	***	*	ns

n = number of replicates. RHIZ⁻: plants inoculated with *Meloidogyne incognita* only; RHIZ⁺: plants inoculated with *Rhizobium etli* and *Meloidogyne incognita*.

*, ** and *** indicate significant differences at $P \leq 0.05$, 0.01 and 0.001, respectively, according to Tukey's equal HSD test; ns: not significant.

inoculated with *R. etli* CNPAF 512. No significant differences in root length were observed. However, greener and more abundant leaves were observed in plants inoculated with *R. etli* CNPAF 512.

Pre-inoculation with *R. etli* CNPAF 512 significantly ($P \leq 0.05$) reduced the number of juveniles and females of *R. similis* but not the number of eggs and males; root necrosis was also significantly ($P \leq 0.05$) reduced

(Table 2). In the simultaneous inoculation assay, the number of eggs, juveniles, females and males of *R. similis*, and root necrosis were significantly ($P \leq 0.05$) reduced in plants inoculated with *R. etli* compared with non-inoculated plants. In the simultaneous inoculation assay, much lower numbers of eggs, juveniles and females, but not males, of *R. similis* were observed compared with the pre-inoculation assay in both non-inoculated plants and

Table 2. Effect of pre-inoculation with *Rhizobium etli* followed 3 weeks later by inoculation with ca 60 females of *Radopholus similis* (pre-inoculation assay) and simultaneous inoculation with both *R. etli* and ca 60 females of *R. similis* (simultaneous inoculation assay) on the reproduction of *R. similis* and root necrosis of common bean plants, grown in an *in vitro* autotrophic tripartite culturing system, at 8 weeks after nematode inoculation.

Treatment	n	Number				Root necrosis
		Eggs	Juveniles	Females	Males	
Pre-inoculation with <i>R. etli</i> followed by inoculation with <i>R. similis</i>						
RHIZ ⁻	8	206	2517	585	125	3
RHIZ ⁺	8	1037	1275	294	161	2
		ns	**	**	ns	**
Simultaneous inoculation of <i>R. etli</i> and <i>R. similis</i>						
RHIZ ⁻	6	44	129	52	306	5
RHIZ ⁺	6	17	39	23	175	3
		**	***	**	*	**

n = number of replicates. RHIZ⁻: plants inoculated with *Radopholus similis* only; RHIZ⁺: plants inoculated with *Rhizobium etli* and *Radopholus similis*.

Root necrosis on a 0 to 7 scale: 0 = absence of lesions; 1 = sporadic lesions and traces of infections in <5% of the roots; 2 = larger coalesced lesions in 5 to up to 10% of the roots; 3 = necrotic root parts present in 10 to up to 25% of the roots; 4 = 25% of roots necrotic and lesions visible in the other roots; 5 = between 25 and up to 50% of roots necrotic and lesions visible in the other roots; 6 = between 50 and up to 75% of roots necrotic and lesions visible in the other roots; 7 = >75% of roots necrotic.

*, ** and *** indicate significant differences at $P \leq 0.05$, 0.01 and 0.001, respectively, according to Tukey's equal HSD test or the Kruskal-Wallis test (root necrosis); ns: not significant.

plants inoculated with *R. etli* CNPAF 512. Nodulation of plants was significantly ($P \leq 0.05$) lower in the pre-inoculation assays with *M. incognita* (both trials) and *R. similis* compared with the simultaneous inoculation assays.

Discussion

The common bean plantlets developed a good root system, rhizobial inoculation resulted in functional nodules, and the infective developmental stages of *M. incognita* and *R. similis* infected the plantlets and developed and reproduced successfully inside the root systems. All these observations validated the *in vitro* autotrophic tripartite culture system as a highly controlled, reproducible and easy to use model system to study plant-rhizobia-endoparasitic nematode interactions. Autotrophic plant growth, in the absence of sugar in the medium, results in normal photosynthetic-active plant tissues, hormonal balance and physiological source-sink relationships, which were considered major limitations of the use of excised organ cultures to study *in vitro* the interactions between plants and either beneficial organisms or pathogens, because they may alter the plant's physiology (Fortin *et al.*,

2002; Voets *et al.*, 2005). Also, this model system presumably allowed the plant to release root exudates in the agar, which specifically induced the synthesis of nodulation factors in rhizobia that initiated the nodulation process (Peters *et al.*, 1986; Pandya *et al.*, 1999) and attracted the infective developmental stages of the plant-parasitic nematodes to the roots (Perry & Curtis, 2013). Finally, it allows monitoring throughout the duration of the experiment plant growth, nodulation and, in the case of root-knot nematodes, also root galling and the emergence of egg masses from the roots.

Both pre-inoculation with *R. etli* CNPAF 512 and simultaneous inoculation significantly suppressed reproduction of the sedentary endoparasitic nematode *M. incognita* on common bean. This result confirms previous observations of the bio-protective suppression of *M. incognita* following either pre- or simultaneous inoculation of leguminous crops (chickpea, mungbean, lentil, pigeonpea) with rhizobia (Fazal *et al.*, 1992; Khan *et al.*, 2018). Both pre-inoculation with *R. etli* CNPAF 512 and simultaneous inoculation also significantly suppressed the reproduction of the migratory endoparasitic nematode *R. similis* on common bean. This result confirmed (Van der Veken *et al.*, data unpubl.) that simultaneous inoculation

of soybean with *R. etli* CNPAF 512 and *R. similis* significantly suppressed nematode reproduction. For both assays with *M. incognita* and *R. similis*, no differences were observed between the bioprotective effect of pre-inoculation with *R. etli* CNPAF 512 and simultaneous inoculation. Nodulation significantly suppressed the number of *R. similis* males in the simultaneous inoculation assay but not in the pre-inoculation assay. For the other developmental stages (eggs, juveniles and females) this was not the case. Males are often rare in root-knot nematode populations but in some species such as *M. incognita* more J2 developed into males under adverse conditions (Jones *et al.*, 2013). It is possible that an unknown stress factor during the simultaneous inoculation assay resulted in the development of more males, and that simultaneous inoculation of *R. etli* CNPAF 512 and J2 of *M. incognita* also resulted in suppression of the number of males that developed.

In contrast with the mechanism involved in the nematode-suppressive effects of arbuscular mycorrhizal fungi (AMF) on plant-parasitic nematodes (Schouteden *et al.*, 2015), the mechanisms involved in the bio-protective effects of rhizobia against plant-parasitic nematodes are much less studied. Competition for nutrients and space between *R. etli* CNPAF 512 and *M. incognita*, aggravated in the confined space in which the root systems of common bean had to develop in our *in vitro* assays, may have played a role in the observed nematode-suppressive effect against this root-knot nematode species. Competition for nutrients is considered a potential mechanism for suppression of plant-parasitic nematodes by endophytic bacteria (Hallmann *et al.*, 1997). In potato, *R. etli* G12, a rhizosphere coloniser with endophytic potential, preferentially colonised the root tips and emerging lateral roots, which were also the preferential penetration sites for the infective J2 of root-knot nematodes (Jones *et al.*, 2013), and approximately 20% of the galls induced by *M. incognita* (Hallmann *et al.*, 2001). Based on the large numbers of *R. etli* G12 that were often observed in the gall tissues, Hallmann *et al.* (2001) suggested that leaked nutrients became available to the endophytic bacteria. Root-knot nematode-induced giant cells inside the galls act as a metabolic sink and contain 4–6 times more glucose and free amino acids than actively growing root tip cells (Huang, 1985). These nutrients were transported from the surrounding tissues into the giant cells and it is possible that endophytic bacteria intercept and take up these nutrients. Fewer nutrients would then become available for the developing females of *M. incognita*, interfering with their development and reproduction. In our assays with *M. incognita*,

it was obvious, based on the high numbers of nodules, that *R. etli* CNPAF 512 was highly successful in colonising the roots of the common bean plantlets. Visual observation of the plantlets showed that the nodules and galls induced by *M. incognita* were often situated closely together. This may have limited the penetration sites available for the infective J2 and the sites inside the roots where the J2 could establish a feeding site. Competition for nutrients between rhizobia and migratory endoparasitic nematodes, such as *R. similis*, has not been reported so far but it cannot be excluded that the mechanisms described above to explain the bio-protective effect of *R. etli* CNPAF 512 against *M. incognita* also apply for *R. similis*. Migratory endoparasites do not establish feeding sites but feed upon the cells of the cortex when migrating through the root system causing leakage of the nutrients inside these cells. However, no *R. similis* was recovered from the nodules so we do not expect competition for nutrients to have played a significant role for the observed nematode-suppressive effect against *R. similis*. Another mechanism that may have played a role in the observed nematode-suppressive effect against both *M. incognita* and *R. similis* was non-pathogenic rhizobacteria-mediated induced systemic resistance (ISR), a systemic resistance in plants that is phenotypically similar to pathogen-induced systemic acquired resistance (SAR; van Loon *et al.*, 1998).

The observation that nodulation of the common bean root systems was significantly lower in the pre-inoculation assays with *M. incognita* (both trials) compared with the simultaneous inoculation assays confirmed numerous previous reports that *M. incognita* infection (and also *R. similis* as demonstrated by our study) may also affect root nodulation by rhizobia (see, for example, Huang, 1987; Sharma & Tiagi, 1990; Khan *et al.*, 2002, 2018). The observation that in both assays with *M. incognita* and *R. similis*, a significantly higher number of nodules had developed on the root systems of the simultaneous inoculation assay compared with the pre-inoculation assay suggests that the simultaneous inoculation of *R. etli* CNPAF 512 and the nematodes may have facilitated, in one way or the other, root penetration and colonisation by the rhizobia, for instance, by rhizobia adhering to the cuticle of the nematodes when these penetrated and migrated through the roots. By contrast, on pea, a greater reduction in the number of nodules was observed when *M. incognita* was inoculated simultaneously with a *Rhizobium* strain compared with when the *Rhizobium* strain was inoculated 2 weeks before *M. incognita* (a reduction of 30 vs 12%, respectively; Sharma & Tiagi, 1990). However, on lentil, no

difference in the number of nodules was observed when a *Rhizobium* strain was inoculated either together with or 10 days before *M. incognita* (Fazal *et al.*, 1992). On soybean, higher numbers of nodules formed on plants infested with *P. penetrans* compared with the non-infested plants after inoculation with *Bradyrhizobium japonicum*; however, when nematodes invaded roots with established nodules the number of nodules was not affected (Elhady *et al.*, 2020).

In both the pre-inoculation and simultaneous assays with both *M. incognita* and *R. similis*, no significant increase in root growth (tap root length) was observed in both trials between the RHIZ⁻ and RHIZ⁺ treatments. However, in the RHIZ⁻ treatment infected with *M. incognita*, nematode infection was more severe (more J2, ELF and egg masses) compared with the RHIZ⁺ treatment, resulting in a lower shoot weight compared with the RHIZ⁺ treatment (data not shown). Thus, *R. etli* CNPAF 512 limited these adverse effects of *M. incognita* on plant growth by suppressing the nematode's reproduction. Most probably, the growth-promoting effect of *R. etli* CNPAF 512 on root growth (tap root length) was limited due to the confined space in which the root systems had to develop in our *in vitro* assays.

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