Plant Soil (2009) 318:117–126
DOI 10.1007/s11104-008-9822-y

REGULAR ARTICLE

Competitiveness of a native *Rhizobium leguminosarum* biovar *trifolii* strain for nodule occupancy is manifested during infection

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Received: 14 July 2008 / Accepted: 27 October 2008 / Published online: 28 November 2008
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Abstract The stages in the nodulation process that determined the competitiveness of *R. leguminosarum* bv. *trifolii* (*Rlt*) strain 20–15, which proved to be highly competitive for nodulation in Iceland fields tests over several years, is analysed. White clover (*Trifolium repens* L.) roots were inoculated with inoculum mixtures containing three strains (*Rlt* 20-15, *Rlt* 8-9 and *Rlt* 32-28) in different proportions and cell densities. Competitiveness in root colonization, formation of infection threads and nodule development was assessed for *Rlt* 20-15 and its weakest competitor, *Rlt* 32-28. ERIC-polymerase chain reaction (PCR) DNA fingerprinting was used to identify inoculated strains recovered from root surfaces and individual nodules. GFP or DsRed tagged strains were used to determine identity in root hairs and nodules. Both strains colonized the root equally at all inoculum ratios tested. But, *Rlt* 20-15 initiated significantly more infection threads and formed more nodules than *Rlt* 32-28. These results show that *Rlt* 20-15 expresses its nodulation competitiveness during infection, either at infection thread initiation or during successive growth in the infection threads. The data presented support earlier observations that this strain competed well in the field in spite of its inferior ability to survive in the soil.

Keywords *Rhizobium leguminosarum* bv. *trifolii* · Competitiveness · Nodule occupancy · Root colonization · Infection threads · *Trifolium repens*

Responsible Editor: Harsh P. Bais.

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Introduction

Rhizobia are soil bacteria that form symbiotic associations with leguminous plant species, with a unique feature of forming root nodules in which the bacteria reduce atmospheric nitrogen to ammonia for assimilation by the host legume (Merrick and Edwards 1995). For more than a century, agricultural practices have tried to take advantage of this symbiosis by inoculating legumes with more effective nitrogen fixing strains with the aim of improving crop growth without

addition of chemical nitrogen fertilisers. However, little success has been achieved from such practices because many strains used as inoculants lack competitive fitness to infect and occupy nodules of target host plants (Brockwell et al. 1995).

Competition for nodulation is a quantitative phenotype, which determines the ability of certain *Rhizobium* strains to dominate in the nodules of a given legume host in competition with other strains present in the root rhizosphere (Dowling and Broughton 1986). This may be a characteristic property of a strain that can be expressed only during interaction with the legume host (Triplett and Sadowsky 1992). As yet, we know very little about the molecular mechanisms that determine the relative competitiveness between the nodulating strains. We also lack a comprehensive understanding of which stage(s) in the nodulation process is most critical for the success or failure of a given strain to occupy the nodule. Several factors have been implicated in competitive nodulation namely motility and chemotaxis, antibiotic production, selective substrate utilisation, faster growth and colonization of the rhizosphere, rate of infection, cell surface determinants and nodule forming efficiency (reviewed in Vlassak and Vanderleyden 1997; Sessitsch et al. 2002). The data generated from studying these factors are however inconsistent and do not hold for all *Rhizobium*-legume systems. For example, while the numbers of rhizobia in the root rhizosphere of soybean (*Glycine max* L.) and annual medics (*Medicago* spp.) positively correlated with higher nodule occupancy (Herridge et al. 1984; Brockwell et al. 1989; Young and Brockwell 1992), no such correlation was found to exist in clover (*Trifolium* spp) by Leung et al. (1994). A more recent study also provided a genetic evidence to show that mutant strains impaired in root colonisation displayed better nodulation competitiveness than their isogenic parental strain on alfalfa (*M. sativa*) roots (Jensen et al. 2005). In addition to genetic traits of the bacteria, many other factors, including the plant genotype, soil pH, temperature, nitrogen concentration, and microbial antagonisms among others, have been shown to influence strain competitiveness for nodule occupancy (Vlassak and Vanderleyden 1997).

Our previous studies showed significant differences in nodule occupancy among three unmarked *R. leguminosarum* biovar *trifolii* (hereafter abbreviated as *Rlt*) strains introduced into a field in Iceland, initially devoid of clover nodulating rhizobia (Svenning et al. 2001). Among these strains, *Rlt* 20-15 consistently occupied 80–90% of the nodules tested; while *Rlt* 8-9 and *Rlt* 32-28 occupied 9–18% and 3%, respectively, of the nodules sampled from white clover (*T. repens* L.). This nodulation competitiveness pattern has been maintained in the field over a decade, and also shown to be consistent across a wide range of clover genotypes including the red clover (*T. pratense* L.) cultivar Betty (Duodu et al. 2007). Analysis of the relative population of these three strains in the field revealed that the high nodule occupancy of the dominating strain (*Rlt* 20-15) in field isolates is due to its greater competitiveness rather than its survival in the soil environment (Duodu et al. 2005). In fact *Rlt* 32-28 was present in much higher numbers in the soil (even in the absence of clover) than *Rlt* 20-15, which appears to be a poor saprophyte.

In this study we have undertaken a systematic in vitro analysis to determine the critical stages in the nodulation process where *Rlt 20*-15 strain's competitiveness is determined. We report the results of the comparison between *Rlt* 20-15 (strongest competitor) and *Rlt* 32-28 (weakest competitor) for competitive root surface colonization, infection thread formation and nodule development under controlled experimental conditions. Since the strains are unmarked, the rep PCR (polymerase chain reaction) fingerprinting technique was used to identify the recovered isolates from root surfaces and nodules in the root colonisation and nodule forming efficiency experiments. Strains were tagged with green and DsRed fluorescent proteins (Gage 2002), to follow their invasion of the root hair inside the infection threads. This study provides evidence to show that greater nodule occupancy by the competitive strain *Rlt* 20-15 results from higher number of infections induced by this strain rather than through better colonisation of the root rhizosphere.

Materials and methods

Plant material and seed surface sterilization

White clover (*T. repens* cv. HoKv 9238) seeds were obtained from Plant Research Centre, Holt, Tromsø. This cultivar is a self incompatible outcrossing polyploid, not clonally propagated and therefore not a genetically uniform population. The seeds were surface sterilised by immersion in 95% ethanol for 10 s, and in 3% (v/v) hydrogen peroxide (Merck,

Whitehouse Station, NJ, USA.) solution for a period of 5 min. To drain off the peroxide, the seeds were rinsed with six changes of sterile distilled water. They were allowed to imbibe in sterile water for 4 h, (Somasegaran and Hoben 1994), and then incubated on 0.9% water agar for germination. Prior to germination, the seeds were pre-conditioned in the dark at 4°C overnight at room temperature.

Rhizobium isolates and preparation of inocula

The three unmarked strains (*Rlt* 20-15, *Rlt* 8-9 and *Rlt* 32-28) used in this study were selected from our natural collections of soil populations from a site in Tromsø. Selections were based on their high nitrogen fixing ability and distinct PCR fingerprint patterns. Pure cultures of these strains were grown to early stationary phase cultures in Tryptone Yeast (TY,) liquid media at 27°C (115 rpm) for 4 d. The cells were harvested by centrifugation (5 min at 10,000 g) at room temperature and washed two times with sterile water. This was followed by determination of the optical densities at 600 nm (OD_{600}) with Spectra Max 250 Molecular Device (Global Medical Instrumentation Inc, Albertville, Minnesota, USA). The actual number of cells in the inoculum for each strain was determined by plating each suspension on TY plates and incubating for 4 d at 27°C.

Nodule occupancy test

In vitro nodule occupancy tests were performed by applying bacterial inoculum at saturating (10^6 per ml or root) and unsaturating (10^2 per ml or root) concentrations. The individual strains were adjusted to the appropriate cell density and mixed in different proportions based on a simplex design (Kirwan et al. 2007). Due to differences in what we intended to add as inoculum and the actual cell counts on plates, the proportional mixtures of cells were corrected for and used in comparison of nodule occupancy by the individual strains. Germinated seedlings were transferred to sterilized Magenta jars (Sigma, St Louis, MO, USA) containing washed vermiculite saturated with Broughton's N-free nutrient solution (Broughton and Dilworth 1970). Vermiculite was washed three times with tap water and soaked overnight in nitrogen-free Broughton's solution. This was then used to fill the jars. Five sterilised seedlings of clover (2 d old) were planted in each jar and grown under supplemented light from Phillips HPI/T 400 W lamps (Philips, Turnhout, Belgium) with 17 h photoperiod at 25°C. Each plant was aseptically inoculated with 1 ml of inoculum mixture. After inoculation, the plants were further incubated in the growth room. Nodulation was assessed 4 weeks after planting. Nodules on the roots were surface sterilized in 70% ethanol and 6% H_2O_2 with a drop of Tween 20 (Merck, Darmstadt, Germany). Preparations of nodule and cell suspensions were as described previously (Svenning et al. 2001).

DNA fingerprinting of isolates

Nodule isolates and crushed nodules were individually screened by ERIC DNA PCR fingerprinting (Versalovic et al. 1991; de Bruijn 1992). The PCR amplification reaction was performed in a total volume of 25 µl containing 1 µl of bacterial cell suspension or crushed nodule as a template, 18 µl of sterile MilliQ water, 2.5 µl of 10 × reaction buffer for DynaZyme polymerase (Finnzymes Oy, Espoo, Finland), 1.0 µl of 50 pmol each of ERIC 1 and ERIC 2 primers (Eurogentec, Seraing, Belgium), 0.5 µl of 10 mM dNTP mix F-560L (Finnzymes Oy, Espoo, Finland) and 1.0 µl of DynaZyme ™ DNA polymerase (2U/µl) F-501, (Finnzymes Oy, Espoo, Finland). The cycling condition was as described by de Bruijn (1992). Twelve micro-litre of the PCR amplified DNA products were loaded onto 1.5% Seakem®LE agarose (FMC BioProducts, Rockland, Me., USA) gels and run at room temperature in Tris-borate-EDTA (TBE) at 80 V for 3 h. Ethidium bromide stained gels were photographed using the Gel Doc™2000 Gel Documentation System (Bio-Rad, Hercules, California, USA). The ERIC patterns were reproducible and individual isolates were classified into distinct ERIC groups based on visual inspection of their fingerprint patterns.

Root colonisation assay

Root colonisation assays were performed according to the method described by Jensen et al. (2005). Briefly, white clover seeds were surface sterilized and germinated as described before. Seedlings were transferred singly to sterilized Magenta jars (Sigma) containing sterile vermiculite and inoculated with 100–200 bacterial cells per root. Each treatment had five plants in triplicate with three jars per replicate. At

seven days after inoculation, roots were harvested under sterile conditions and sonicated for 10 min in ice-cold buffer (0.05 M phosphate [pH 7.2] containing 0.01% (wt/vol) Tween and vortexed for 30 s at maximum speed. Appropriate dilutions of these suspensions were plated out onto TY medium and the individual cells recovered from the root surface were screened by ERIC PCR DNA fingerprinting (de Bruijn 1992). Experiments were repeated at least twice.

Nodule efficiency test

Sterilised pre-germinated clover seedlings (2 d old) were transferred from water agar plates to plastic growth pouches (North-rup King Seed Co., Minneapolis, MN). Each pouch contained 5 plants, with 10 pouches per treatment. The pouches were moistened with 10 ml N-free Broughton's nutrient solution. In order to prevent drying and to maintain sterility, seedlings in growth pouches were placed very close together in boxes and covered with aluminium foil until inoculation. Roots of individual seedlings were inoculated 3 d after transfers to pouches with 100 µl of an appropriate dilution of the bacterial suspension by dripping slowly on its surface. Prior to inoculation, the position of the root tip (RT) and the smallest emerging root hairs were marked under a dissecting microscope (Bhuvaneswari et al. 1981). Plants were grown in a growth chamber at temperature of 25°C, with a photoperiod of 17 h. The pouches were moistened with sterile nitrogen free Broughton's solution as required. The number and relative location of nodules on the main roots were determined 10 d after inoculation using a dissecting microscope. Experiments were repeated at least twice with 100 replicate plants in each treatment.

Construction of GFP and DsRed marked *Rlt* strains for infection studies

The two *Rlt* strains (strain 20-15 and strain 32-28) were cross tagged with GFP and DsRed fluorescent proteins by mating plasmids pDG70 or pDG77 (Gage 2002) into these strains by tri-parental mating as described by Jensen et al. (2005). All bacteria used in mating were grown to late log phase. Transconjugants were selected on TY medium plates containing polymyxin (20 µg/ml) and tetracycline (5 µg/ml). Mutant strains expressing GFP and DsRed proteins gave green and red fluorescence, respectively, under fluorescence microscope. The stability of plasmids in the absence of antibiotics was tested by growing the GFP and DsRed marked strains through successive generations in TY liquid at 28°C, and plating serial dilutions of cultures on non-selective plates. Plasmid loss was scored by counting the number of colonies that showed no green or red fluorescence. The marked strains were inoculated and re-isolated from nodules to check for plasmid stability during symbiosis. We also compared the growth rate of the fluorescent tagged and unmarked strains by periodically measuring the optical density (OD) at A_{600} over 4 d (data not shown).

Competition at root-hair infection stage by GFP and DsRed marked strains

Clover seedlings were established in Magenta jars and in growth pouches as described above. Plant roots were inoculated with 1:1 mixture of the marked strains at a density of 10^5 cells per Magenta jar or growth pouch. Conditions of plant growth were as described before. The number of infection threads and nodules expressing GFP and DsRed on each root were scored after a minimum of 10 d. In separate experiments, plants were inoculated with 1:1 mixture of GFP tagged and untagged wild-type strains and nodules were allowed to develop for 21–30 d. Selected nodules were surface sterilised, crushed and observed for green fluorescent bacteria under the microscope.

Microscopy

Plants roots inoculated with fluorescent marked bacteria were mounted in de-ionized water on a cover slip and observed under a Leitz DAS (DM RB/E) microscope equipped with epifluorescence detection. Filter sets tailored to the specific chromophores were used. GFP expression was recorded using the GFP filter cube with BP470/40 excitation filter and BP525/50 suppression filter. DsRed fluorescence was monitored using the excitation filter BP515-560 and suppression filter LP590. Images were obtained using confocal laser scanning microscope (LSM510 META, Carl Zeiss, Germany). GFP was excited using the 488 nm laser line and emission detected using the 500 to 530 nm band pass filter. DsRed was excited using the 543 nm laser line and emission detected using the 560 nm long pass filter.

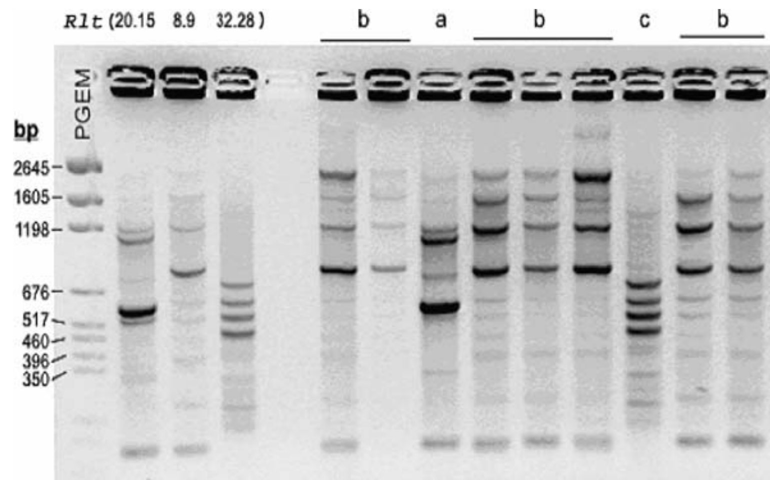

Fig. 1 ERIC-PCR fingerprint patterns generated from cells and crushed nodule suspensions showing nodule occupancy of tested strains on white clover. *Rlt* (20-15, 8-9 32-28) are the standard reference patterns of the three tested strains. a, b and c represent identified patterns from crushed nodules following inoculation of plants with mixtures of the three strains. pGEM is a Molecular marker, (Promega)

Statistical analysis

We examined the number of nodules infected by each strain and tested for deviations from the proportion of each strain present initially in the inoculum using a multinomial base-line category analysis (Agresti 2002). The competitiveness of each strain is expressed as the proportion of nodules occupied by the strain relative to the initial proportion of that strain in the inoculum.

Significant differences between treatment means of the root colonisation and infection data were deduced by Chi-square tests (Wardlaw 2000) using the MINITAB statistical program (Minitab Inc., Coventry, U.K.).

Results

The nodulation competitiveness of the three *Rlt* strains was assessed at different inoculum combinations and cell densities under controlled conditions. The ERIC fingerprints of the strains for identification are shown in Fig. 1. The analysis showed a significant change in composition from the initial proportion of each strain in the inoculum to their final nodule occupancy (Fig. 2). Even though there was a general tendency for higher nodule occupancy by each strain with increase in cell numbers, *Rlt* 20-15 consistently had a greater relative nodule occupancy than expected (i.e. had a relative value greater than 1 for all treatments) (Table 1), *Rlt* 32-28 failed to achieve its expected nodule occupancy in all inoculum combinations tested (values less than 1 in Table 1), *Rlt* 8-9 on the other hand, showed an inconsistent pattern of change, with some relative values greater than expected but others less than expected (Table 1). At non-saturating levels of 10^2 cells per root (where bacteria have to competitively colonize the root

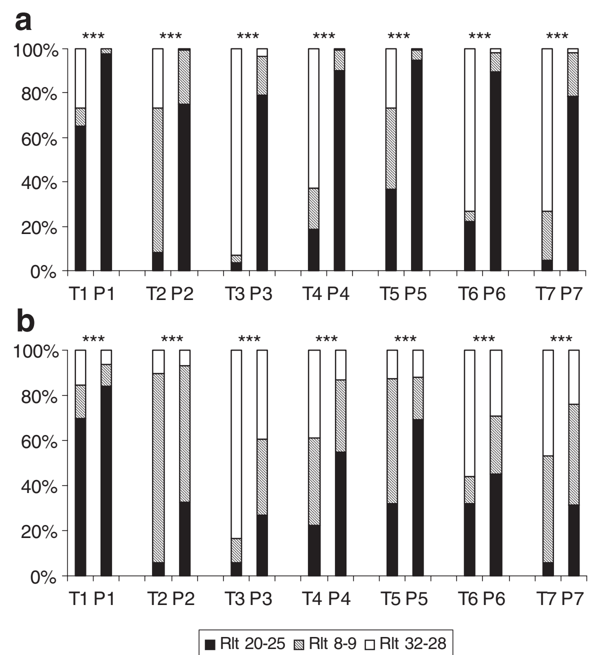

Fig. 2 Competition for nodule occupancy on white clover by the individual *Rlt* strains at (a) a non saturating inoculum density of 10^2 cells per root and (b) a saturating inoculum density of 10^6 cells per root. T1-7 represents the proportions of each strain present in the initial inoculum mixtures. P1-7 represents the proportion of nodules each strain infected. Significant differences between the proportions in T and P are indicated by stars above the pair of bars for each density by initial inoculum mixture proportion combination (* $p<0.1$; ** $p<0.05$; *** $p<0.01$)

Table 1 Change in strain composition

Initial Density	Initial proportion in the inoculum for each strain			Proportion of nodules infected relative to the initial proportion		
	Rlt 20-25	*Rlt* 8-9	*Rlt* 32-28	*Rlt* 20-25	*Rlt* 8-9	*Rlt* 32-28
10^2 cells/ root	0.65	0.08	0.27	1.52	0.19	0.01
	0.08	0.65	0.27	10.03	0.28	0.01
	0.03	0.03	0.93	18.47	9.59	0.02
	0.19	0.19	0.63	5.02	0.29	0.01
	0.36	0.36	0.27	2.67	0.06	0.01
	0.22	0.05	0.73	4.39	0.64	0.01
	0.05	0.22	0.73	14.12	1.33	0.03
10^6 cells/root	0.70	0.15	0.15	1.18	0.85	0.33
	0.06	0.84	0.10	5.76	0.72	0.55
	0.06	0.10	0.84	6.63	2.62	0.39
	0.22	0.39	0.39	2.52	0.91	0.23
	0.32	0.56	0.12	2.02	0.58	0.27
	0.32	0.12	0.56	2.25	1.28	0.22
	0.06	0.47	0.47	4.66	1.23	0.31

The three columns in the left hand panel show the initial proportions of the three strains for fourteen experimental treatments. The three columns in the right hand panel show the estimated final proportion of total nodules occupied by each strain relative to this initial proportion. A value of 1 indicates no change from initial proportion, while a value greater (less) than 1 indicates that the strain competed well (poorly). Composition shifted significantly during the course of the experiment for all treatments ($p<0.01$)

surface before nodulating), *Rlt* 20-15 formed most of the nodules and *Rlt* 32-28 formed the least. When the strains were inoculated at saturating concentrations of 10^6 cells per root, there was a decrease in the relative dominance of *Rlt* 20-15, while the relative nodule occupancy of *Rlt* 32-28 increased.

The strong nodulation dominance of *Rlt* 20-15 at low inoculum application, suggested that either it multiplies rapidly on the host root rhizosphere or needs a fewer number of cells to initiate infections. Likewise the increase in relative nodule occupancy of Rlt 32-28 at saturating inoculum concentrations suggests that *Rlt* 32-28 might have competed poorly at colonization stage. To test for colonization, we examined the ability of *Rlt* 32-28 to compete against *Rlt* 20-15 for growth on the plant roots as described in Materials and Methods. The results indicated no significant differences between the two tested strains in root colonization either in competitive or in non-competitive situations (Table 2). When nodule initiation efficiency of these strains were examined (Fig. 3), the nodulation response curve was nearly linear up to 10^5 inoculum

Table 2 Competitive root colonization of white clover roots between *Rlt* 20-15 and *Rlt* 32-28

Strain mixture ratio (*Rlt* 20-15/*Rlt* 32-28)	Total cells [a]	*Rlt* 20-15 cells [a]	*Rlt* 32-28 cells [a]	*Rlt* 20-15 in total (%)[b]	
				Expected	Observed
1:1	65.5 (6.3)	34.2 (4.9)	31.3 (5.5)	50	52a
1:9	92.3 (18.2)	9.0 (2.6)	83.3 (19.2)	11	9.8a
1:0	65.0 (12.1)	NA	NA	NA	NA
0:1	66.7 (12.0)	NA	NA	NA	NA

[a] The numbers represent average bacterial cells per root (10^4) of 90 plants from two experiments. The standard deviations are given in parentheses.

[b] Treatments followed by the same letter are not significantly different at the 95% confidence level.

NA, not applicable

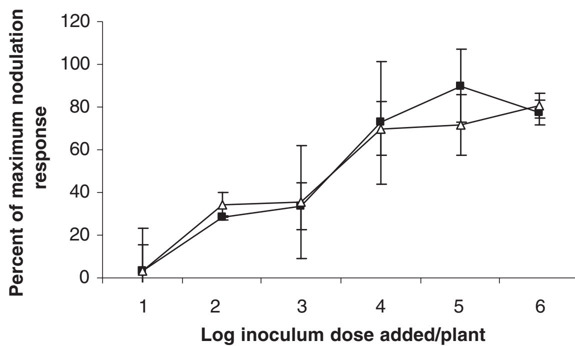

Fig. 3 Inoculum dose-nodulation response of *Rlt* 20-15 (■) and *Rlt* 32-28 (Δ) with white clover. Plants were inoculated with 100 µl of bacterial suspension at the indicated dosage per plant. Nodules on primary root above the root tip mark were determined 10 days after inoculation. Data are shown as means±SD from two experiments with each point representing averages from 90–100 plants

levels, but declined at higher inoculum dosages for *Rlt* 20-15. On the other hand, the nodulation response of *Rlt* 32-28 maintained a more or less linear fashion, and did not reduce at inoculum density above 10^5 bacterial cells. Both strains, however, required the same number of cells to generate half-maximal nodulation response, suggesting that they are equally efficient in initiating nodule formation.

In order to define the stage of nodulation where the competitiveness of strain 20-15 occurs, we followed infection of the root hair *in vivo* with *Rlt* 20-15 marked with GFP fluorescence protein and *Rlt* 32-28 marked with DsRed fluorescence protein and vice versa. Prior to this investigation, we compared nodulation of the fluorescent marked strains with their parental strains. When these were tested at 1:1 inoculum ratio, both marked and parent strains formed similar numbers of nodules that were pink in colour (data not shown). This indicates that the insertion of plasmids expressing the fluorescent proteins did not alter the symbiotic properties or competitiveness of the parental strains. No growth differences were observed between the marked and unmarked strains based on their mean generation times (data not shown). In some of these competition experiments the fluorescent tags were reversed for the strains used. Regardless of the fluorescent tags used, *Rlt* 20-15 significantly formed more infection threads and nodules than *Rlt* 32-28 when co-inoculated onto the plant root. Of the 34 roots screened, 76.1 % (217 out of 285 infection threads observed) of the infections were formed by *Rlt* 20-15, and the rest by *Rlt* 32-28. Among the 302 nodules screened, 87.1 % were occupied by *Rlt* 20-15 and 12.9% by *Rlt* 32-28. We did not observe any infection threads or nodules occupied by both strains (Fig. 4).

Discussion

The goal of this study was to understand the basis for the differential competitiveness of three defined strains of *Rhizobium* on their plant hosts by specifically analysing for the critical stage(s) in the nodulation process where the competition has most likely occurred. Results presented in Table 2, indicates that differences in the initial multiplication and colonisation of the root surfaces is not the cause for differential nodulation competitiveness of the three strains tested. Our results are consistent with other reports from earlier studies (Leung et al. 1994). We tested these three strains *in vitro* for specific bactericidal or bacteriostatic effect towards each other and found that none of the three tested strains inhibited the growth of the others (data not shown). Equal colonization of the root by *Rlt* 20-15 and 32-28 suggests that the competitiveness of the *Rlt* 20-15 is expressed after root colonisation stage, possibly inside the root hair. This suggestion is supported by data from our root hair infection studies where the most competitive strain (*Rlt* 20-15) formed significantly higher number of infections than the least competitive strain (*Rlt* 32-28), and relative nodule numbers more or less corresponded to the relative number of infections for *Rlt* 20-15. On the other hand, about 25% of the infection threads observed and 13% of the nodules screened were occupied by *Rlt* 32-28. The competitiveness of *Rlt* 20-15 can be expressed either at infection thread initiation or during bacterial growth in the infection threads. Similar observations have recently been reported from competition studies between wild-type and trehalose utilization mutant strains on alfalfa roots (Jensen et al. 2005). Since rhizobia are not mobile in the infection thread, the rate of multiplication of bacteria inside the infection thread determines the rate of infection thread proliferation (Gage 2002) and subsequent nodule occupancy. Processes that contribute to improved fitness of the bacteria inside the infection thread therefore may contribute to enhanced nodulation competitiveness.

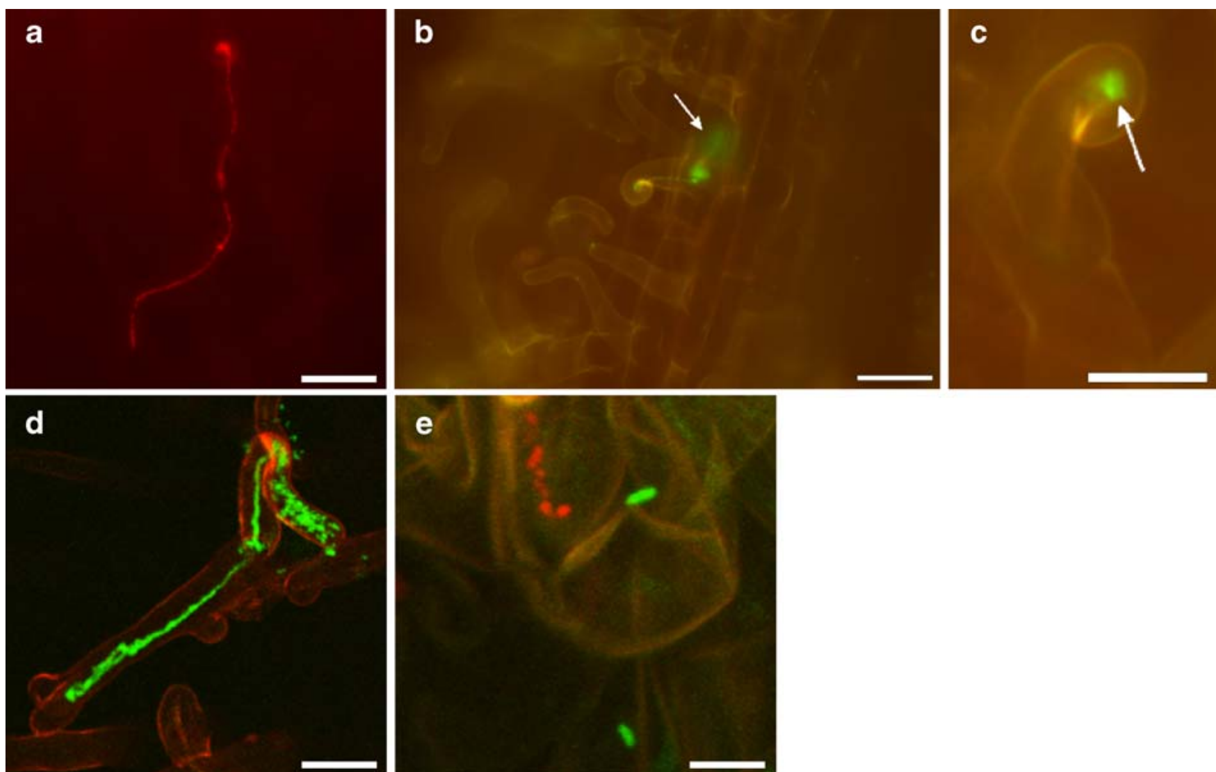

Fig. 4 Examples of infection events following coinoculation of white clover roots with red- and green-fluorescent tagged *Rlt* strains. **a** Infection thread containing DsRed-expressing *Rlt* 32-28. **b** Infection thread leading to a nodule in the inner root tissue (arrowed) containing GFP-expressing *Rlt* 20-15. **c** Curled root hair with green microcolonies (arrowed) induced by GFP-expressing *Rlt* 32-28 that failed to give rise to an infection thread. **d** Infection thread colonised by GFP-expressing *Rlt* 20-15 in a root hair **e** Single cells of GFP- expressing *Rlt* 32-28 and a short infection thread colonised by DsRed expressing *Rlt* 20-15, on the root surface and inside the root hair, respectively. Bar 50 µm

At present, the environmental conditions inside the infection thread determining the multiplication and growth of rhizobia in the infection thread are not known. It is shown that active growth of the symbiont occurs only near the tip of the infection threads in a very small zone and that rhizobial strains that catabolize specific substrate(s) in the infection thread may grow faster and initiate the nodule primordia quickly (Gage 2002). Therefore, we contend that stains with improved competitiveness, may grow more rapidly in the infection thread, to initiate and infect the nodule primordia faster. Since there is a stringent regulation of nodule numbers by the host, such competitive growth might block less competitive ones from completing the infection events and occupying the nodule. The observation that the number of nodules occupied by *Rlt* 32-28 corresponds to only about 50% of infection threads supports this hypothesis.

Generally, rhizobia are exposed to different stress factors during early interactions in symbiosis with the host plant. In alfalfa infections, *S. meliloti* mutants with impaired mechanism to cope with oxidative stress are unable to multiply in the infection threads (Herouart et al. 2002). Recently, Jensen et al. (2005) demonstrated that *S. meliloti* is exposed to osmotic stress in the infection threads, and mutant strains with ability to accumulate trehalose in their cells enhanced their nodulation competitiveness via increased number of infections. Also, it was shown that ethylene negatively regulates rhizobial infection and nodulation (Zaat et al. 1989; Okazaki et al. 2004), and strains with greater ability to overcome ethylene stress during infection showed enhanced nodulation competitiveness (Ma et al. 2004). Taken together, these observations suggest that strains with better stress adaptation may show greater competitiveness for nodule occupancy. Whether the competitiveness of *Rlt*

20-15 is related to its better stress coping ability during growth inside the root hair is yet to be determined.

The results presented in this study show that the competitiveness of *Rlt* 20-15 is not due to its superior root colonization but may be related to its ability to grow better in the infection threads. This strain is among few native strains that maintain similar levels of nodulation competitiveness in the laboratory and under certain field conditions, and may be useful as a model organism for an in-depth analysis of microbial competition and to study the factors that influence growth inside the infection threads.

Acknowledgements This work was supported by the Norwegian Research Council grant (126312/110), as part of NKJ (Nordic Joint Committee for Agricultural Research) project 106- Exploring legume-*Rhizobium* symbioses for sustainable agriculture. Travel support to Ireland for the statistical analysis was funded by COST 852 short-term scientific missions. We thank Dr. Daniel J. Gage at the University of Connecticut, USA, for kindly providing us with the GFP and DsRed plasmids. Coby Weber is thanked for her technical assistance.

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