Rapid report

The model legume *Medicago truncatula* A17 is poorly matched for N_2 fixation with the sequenced microsymbiont *Sinorhizobium meliloti* 1021

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Summary

• *Medicago truncatula* (barrel medic) A17 is currently being sequenced as a model legume, complementing the sequenced root nodule bacterial strain *Sinorhizobium meliloti* 1021 (Sm1021). In this study, the effectiveness of the Sm1021–*M. truncatula* symbiosis at fixing N_2 was evaluated.

• N_2 fixation effectiveness was examined with eight *Medicago* species and three accessions of *M. truncatula* with Sm1021 and two other *Sinorhizobium* strains. Plant shoot dry weights, plant nitrogen content and nodule distribution, morphology and number were analysed.

• Compared with nitrogen-fed controls, Sm1021 was ineffective or partially effective on all hosts tested (excluding *M. sativa*), as measured by reduced dry weights and shoot N content. Against an effective strain, Sm1021 on *M. truncatula* accessions produced more nodules, which were small, pale, more widely distributed on the root system and with fewer infected cells.

• The Sm1021–*M. truncatula* symbiosis is poorly matched for N_2 fixation and the strain could possess broader N_2 fixation deficiencies. A possible origin for this reduction in effectiveness is discussed. An alternative sequenced strain, effective at N_2 fixation on *M. truncatula* A17, is *Sinorhizobium medicae* WSM419.

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Introduction

The *Medicago* genus is one of prime importance to agriculture because of the ability of its members to fix atmospheric N_2 in symbiosis with bacteria. *Medicago sativa* (alfalfa, lucerne), arguably the most widely cultivated species, has been studied extensively, often in conjunction with the microsymbiont *Sinorhizobium meliloti* 1021 (Sm1021). The sequencing of the genome of Sm1021 (Galibert *et al.*, 2001) highlighted a dearth of knowledge of host genetic determinants, making sequencing of the host genome a necessary next step. *Medicago sativa* was poorly suited to this role and the more

amenable *Medicago truncatula* (barrel medic) was chosen (Barker *et al.*, 1990; Cook, 1999). The Sm1021–*M. truncatula* symbiosis has now emerged as the model system for studying indeterminate nodulation, and the sequencing of the *M. truncatula* genome continues (Young *et al.*, 2005).

A broad range of effectiveness of symbiotic interactions exists in legume symbioses (Sprent, 2007). Howieson *et al.* (2005) have classified these interactions into four groups.

- No symbiotic interaction, where no infection of the host occurs.
- A parasitic interaction where nodule-like bodies form, but no N_2 is fixed.
- A partially effective symbiosis where fixation occurs, but at a rate between 20 and 75% of the nitrogen (N)-fed control.
- An effective symbiosis where biomass and N accumulation occur at levels 75% or higher, relative to the N-fed control.

At present, there are few published data quantifying N_2 fixation between the model host *M. truncatula* and Sm1021. However, data are available for *S. meliloti* SU47 (Brockwell & Hely, 1966; Snyman & Strijdom, 1980), the parent strain of Sm1021 (Meade *et al.*, 1982). Here, we provide evidence that the symbiosis between the model legume *M. truncatula* and the sequenced strain Sm1021 is poorly matched for N_2 fixation.

Materials and methods

Bacterial strains and plant accessions

Sinorhizobium meliloti 1021 was obtained from Professor Sharon Long (Stanford University, USA). *Sinorhizobium medicae* WSM419 (Centre for *Rhizobium* Studies WSM Genebank) is an isolate from Sardinia (Italy; Howieson & Ewing, 1986) and was the commercial inoculant for annual *Medicago* spp. in Australia from 1985 to 1993 (Bullard *et al.*, 2005). *Sinorhizobium meliloti* WSM1022 is a field isolate obtained from Naxos (Greece) from the annual legume *Medicago orbicularis* (J. G. Howieson, CRS, WSM Genebank).

Medicago truncatula accessions SA1619 (cv. Jemalong), SA27783 (cv. Caliph) and SA37443 (A17, the reference line for the genome sequencing effort), *Medicago arabica* SA36043, *Medicago littoralis* SA421 and *Medicago tornata* SA3639 were obtained from the Genetic Resource Centre, SARDI (South Australian Research and Development Institute, Adelaide, South Australia). *Medicago sativa* cv. Sceptre, *Medicago polymorpha* cv. Santiago, *Medicago murex* cv. Zodiac and *Medicago sphaerocarpus* cv. Orion, were obtained from the Department of Agriculture and Food, Western Australia.

Experiments to assess N_2 fixation

Plants were grown in free-draining pots in a 1:1 mix of yellow sand and washed river sand, as described by Howieson *et al.* (1995), where growth of legumes is limited by N deficiency, except when they are effectively nodulated. Seeds were scarified, surfaced sterilized in 70% (v/v) ethanol (1 min) and 4% (w/v) NaOCl (3 min), rinsed six times in sterile water and placed onto 0.9% (w/v) water agar plates to germinate. When radicles had emerged, six seedlings were sown aseptically into each pot. Inoculant bacteria were cultured on half lupin-agar (Howieson *et al.*, 1988) for 3 d at 28°C. Cultures were suspended in 1% (w/v) sucrose solution at 10^8 cells ml^{-1} and 1 ml of this suspension was inoculated onto each seedling at sowing. Plants were thinned to four per pot after 2 wk. Control plants were grown in identical conditions but not inoculated. Plus N controls were fed weekly with 5 ml of 0.1 M KNO_3 and negative controls received no N. All plants were given regular nutrients and sterile water as required (Howieson *et al.*, 1995). Three replicates were prepared for each treatment and, within each experiment pot, position was randomly allocated.

Plant shoots were excised 42 d postinoculation and dried for 2 d at 60°C, then weighed. Roots were carefully washed free of soil and were assessed for nodule number, distribution and morphology. Nodules were excised, sectioned, stained and examined as previously described (Yates *et al.*, 2007). Effective, partially effective and ineffective symbioses were determined using the criteria established by Howieson *et al.* (2005). Shoot N content was determined on a Leco F528 Nitrogen Analyzer (CSBP Soil and Plant Laboratory, Perth, Australia).

Experiment 1 investigated the potential for N_2 fixation with three strains of *Sinorhizobium* (WSM419, WSM1022 and Sm1021) inoculated separately onto five annual species of *Medicago.* Experiment 2 investigated N_2 fixation with Sm1021 and WSM1022 on three *M. truncatula* accessions (SA1619, SA27783 and SA37443), as well as the closely related *M. littoralis* and *M. tornata*. Experiment 3 investigated N_2 fixation and nodule morphology produced by three strains of *Sinorhizobium* inoculated separately onto the perennial medic *M. sativa* in comparison with *M. truncatula* SA37443.

Statistical analysis

Experiments were analysed using the analysis of variance (ANOVA) package of GENSTAT version 9. For each plant species/accession, significant differences between treatments were determined by one-way ANOVA followed by a post hoc Fisher's least significant difference (LSD) test with $P < 0.05$. Data for experiments 1 and 2 are presented as a percentage of the N-fed control and were $\log_{10}$ transformed before the statistical analysis.

Results

N_2 fixation across a broad range of *Medicago* hosts

The effectiveness of Sm1021, WSM1022 and WSM419 on a selection of *Medicago* hosts was assessed through measurement of plant production (dry weight of shoots) in a growth environment limited by available N. WSM1022 and WSM419

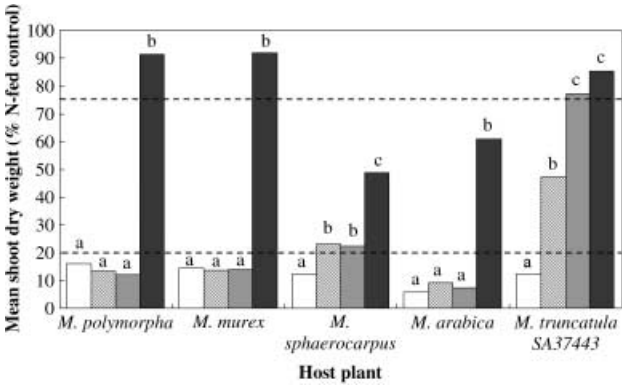

Fig. 1 Plant shoot dry weights, expressed as a percentage of the nitrogen (N)-fed control after growth for 42 d in free-draining pots. Plants were either uninoculated (open columns) or inoculated separately with cultures of *Sinorhizobium meliloti* 1021 (Sm1021; hatched columns), *S. meliloti* WSM1022 (grey columns) or *Sinorhizobium medicae* WSM419 (black columns). Dotted lines delineate effective (≥ 75%), partially effective (between 20 and 75%) and ineffective (≤ 20%) symbioses. Inoculation of *Medicago murex* with either Sm1021 or WSM1022 failed to elicit nodules. Within each plant species, treatments that share a letter are not significantly different according to Fisher's least significant difference (LSD) test ($P < 0.05$).

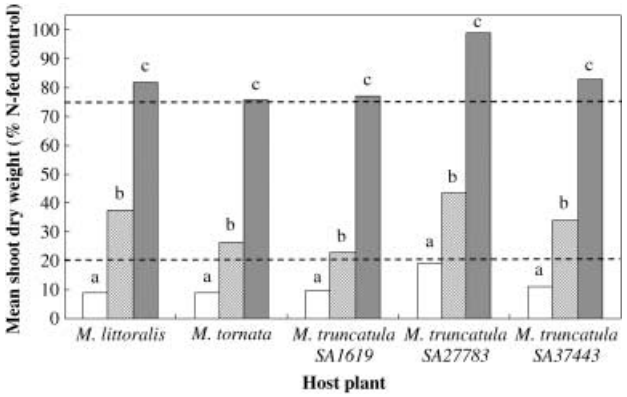

Fig. 2 Plant shoot dry weights, expressed as a percentage of the nitrogen (N)-fed control after growth for 42 d in free-draining pots. Plants were either uninoculated (open columns) or inoculated separately with cultures of *Sinorhizobium meliloti* 1021 (Sm1021; hatched columns) or *S. meliloti* WSM1022 (grey columns). Dotted lines delineate effective (≥ 75%), partially effective (between 20–75%) and ineffective (≤ 20%) symbioses. Within each plant species accession, treatments that share a letter are not significantly different according to Fisher's least significant difference (LSD) test ($P < 0.05$).

inoculated onto *M. truncatula* out-yielded the Sm1021–*M. truncatula* symbiosis by more than 50% ($P < 0.05$). The two former strains were effective with the model host whereas Sm1021 achieved only partial effectiveness (Fig. 1). Sm1021 and *S. meliloti* WSM1022 did not differ in symbiotic characteristics with four other hosts, with both strains

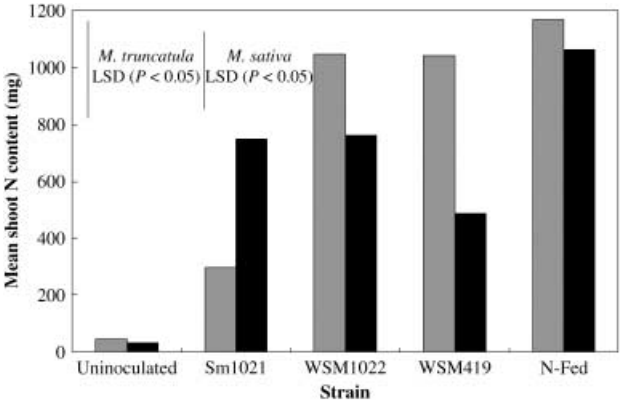

Fig. 3 Mean shoot nitrogen content determined from plants grown in free-draining pots for 42 d. Both *Medicago truncatula* SA37443 (grey columns) and *Medicago sativa* cv. Sceptre (black columns) were either uninoculated or inoculated separately with cultures of *Sinorhizobium meliloti* 1021 (Sm1021), *S. meliloti* WSM1022 or *Sinorhizobium medicae* WSM419. Least significant difference (LSD) ($P < 0.05$) was 380 for *M. truncatula* and 295 for *M. sativa*. N-Fed, nitrogen fed.

failing to fix N_2 on *M. polymorpha*, *M. murex* and *M. arabica*, whilst fixing very poorly on *Medicago sphaerocarpus* (Fig. 1). By contrast, *S. medicae* WSM419 was either effective (*M. polymorpha*, *M. murex*) or partially effective (*M. sphaerocarpus*, *M. arabica*) on these same hosts (Fig. 1).

The partial effectiveness displayed by Sm1021 on *M. truncatula* was also evident from a broader comparison of other compatible hosts (Fig. 2). Whereas WSM1022 fixed effectively with hosts *M. littoralis* and *M. tornata*, as well as with two additional accessions of *M. truncatula*, Sm1021 was suboptimal for N_2 fixation across these hosts (Fig. 2).

Plant shoot N content revealed that N_2 fixation by Sm1021, WSM1022 and WSM419 was not significantly different on *M. sativa* (Fig. 3). Consistent with experiments 1 and 2, Sm1021 on *M. truncatula* showed a significantly lower ($P < 0.05$) shoot N content than either WSM1022 or WSM419 (Fig. 3). Analysis of the plant dry weights (data not shown) confirmed the shoot N-content data.

Nodule development

Average nodule numbers on *M. sativa* inoculated separately with strains Sm1021, WSM419 or WSM1022 did not differ significantly, ranging from five to eight per plant. These nodules were relatively large, dark pink and tightly distributed in the upper root zone (Fig. 4). On *M. truncatula*, nodule number, distribution and morphology were not different when inoculated separately with either WSM1022 or WSM419. By contrast, *M. truncatula* inoculated with Sm1021 showed greater numbers of nodules ($P < 0.05$), ranging from 15–18 per plant. These nodules were smaller

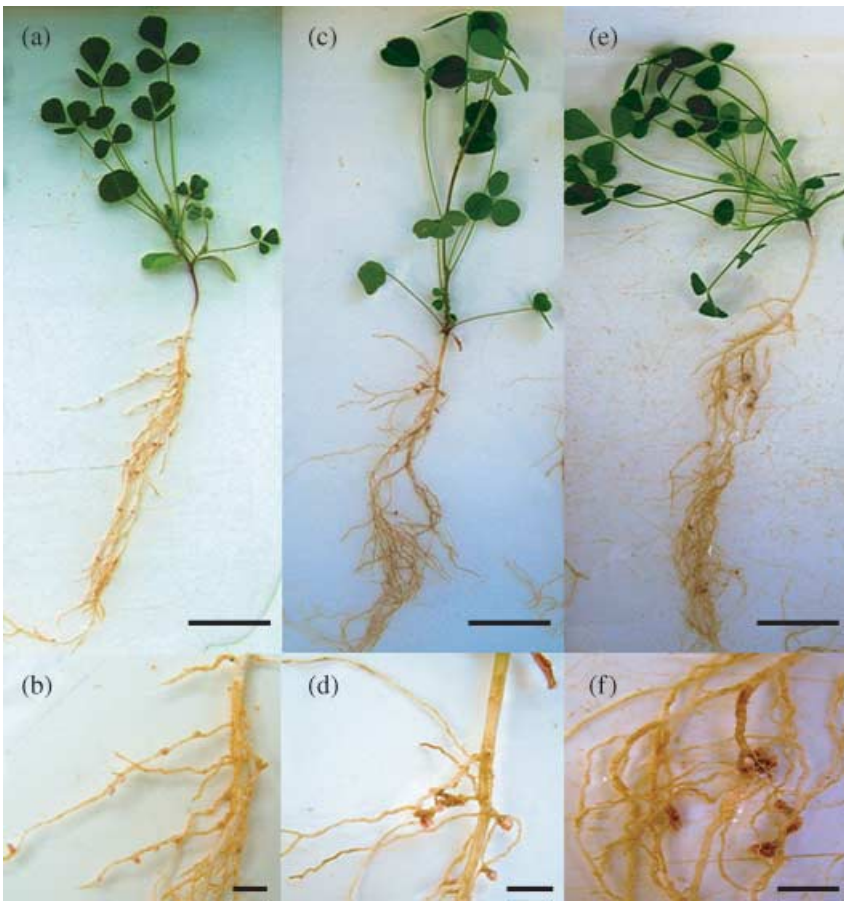

Fig. 4 Photographs of nodule morphology and distribution when *Sinorhizobium meliloti* 1021 (Sm1021) was inoculated onto *Medicago truncatula* (a, b), *Medicago sativa* (c, d) and when *Sinorhizobium medicae* WSM419 was inoculated onto *M. truncatula* (e, f). Bars: (a, c, e), 1 cm; (b, d, f), 500 μm.

and paler, distributed across the entire root system and green at the proximal end (Fig. 4).

Nodule sections revealed that although plant cells did contain bacteroids, there were fewer plant cells with bacteroids in the Sm1021–*M. truncatula* symbiosis than in *M. truncatula* inoculated with either WSM1022 or WSM419. Starch granules were also evident at the plant cell periphery as was a marked occlusion of the interstitial spaces (data not shown). These phenomena were not visible in any effective symbiotic partnership examined.

Discussion

There has been widespread study of the Sm1021 symbiosis with *M. sativa*, leading to an accumulation of genetic, symbiotic and biochemical data on these organisms and culminating in the genome sequencing of Sm1021 (Galibert *et al.*, 2001). *Medicago sativa* was rejected for sequencing, in part because of its large genome, tetraploidy and out-crossing requirement, and *M. truncatula* was chosen instead (Barker *et al.*, 1990; Cook, 1999). Recent research has indicated that the accession selected (A17) carries a chromosomal translocation, which results in the semisterility of pollen in hybrids (Kamphuis *et al.*, 2007). The effect on symbiotic performance is unknown.

We know of no published data on the effectiveness of *M. truncatula* with Sm1021 at N_2 fixation. Simsek *et al.* (2007) have noted that this symbiosis 'was much less efficient ... than the other compatible strains', an observation supported by Garau and co-workers (2005). However, data are available showing the effectiveness of *M. truncatula* with the parent strain SU47 (Brockwell & Hely, 1966; Snyman & Strijdom, 1980); both papers report the symbiosis to be relatively effective, although only Brockwell & Hely (1966) have compared dry weights with an N-fed control.

In the present study, plant dry weight, N content and nodule data together provide strong evidence for a poorly matched symbiosis between Sm1021 and *M. truncatula*. The larger number of nodules on the roots of *M. truncatula* and their atypical morphology are indicative of ineffective nodulation (Mishustin & Shil'nikova, 1971; Frederick, 1978); cobalt and molybdenum deficiency, both of which compromise N_2 fixation, also produce high nodule numbers (Anderson & Spencer, 1950; Riley & Dilworth, 1982). The lower dry weights produced by Sm1021 on *M. littoralis* and *M. tornata* relative to WSM1022 suggest that there may be

broader symbiotic deficiencies in the former strain. That Sm1021 fixed more effectively on *M. sativa* reflects the historical utilization of its parent SU47 as a commercial inoculant with *M. sativa* (Bullard *et al.*, 2005) and the common use thereafter of Sm1021 in the laboratory.

Why is it that SU47 appears to be more effective on *M. truncatula* than Sm1021? SU47, first isolated in 1939 (Vincent, 1941) from *M. sativa* growing at Bathurst, Australia, has, over the years, been sent to many research laboratories around the world and presumably cultured and stored under varying conditions. It is difficult to say how similar the parent strain isolated 62 yr ago is to the spontaneous Str^R strain Sm1021 (Meade *et al.*, 1982) sequenced in 2001 (Galibert *et al.*, 2001), but it is possible that the strain acquired altered symbiotic characteristics in the intervening years. The difference in calcium-spiking behaviour between SU47 derivatives Sm1021 and Sm2011 (Wais *et al.*, 2002) with *M. truncatula* root hairs may also be the result of long-term cultivation.

Researchers working on legumes need to be aware that the Sm1021–*M. truncatula* A17 symbiosis is not optimally matched for N_2 fixation. Molecular analyses of symbiotic requirements need a reliable benchmark against which to make comparisons. The poor effectiveness of the Sm1021–*M. truncatula* symbiosis suggests that it may not be able to fulfil this role. The recently sequenced *S. medicae* WSM419 (Genbank accession NC_009636), shown here to be a better microsymbiont for *M. truncatula* than Sm1021, offers those studying host–strain interactions the opportunity to work on a highly effective symbiosis.

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