

Rhizobial mediation of *Acacia* adaptation to soil salinity: evidence of underlying trade-offs and tests of expected patterns

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Summary

1. The ecological importance of host–soil symbiont associations for plant community structure and dynamics has been recently highlighted, particularly with regard to ecological and evolutionary responses along environmental gradients.
2. We used a range of native Australian shrubby legumes (*Acacia* spp.) and associated root-nodule forming bacteria (rhizobia) in laboratory and glasshouse studies to investigate the ecology of *Acacia*–rhizobial interactions with respect to soil salinity, a major environmental stressor in many parts of the world.
3. Analysis of laboratory growth data provided clear evidence of a trade-off in that growth rate of individual rhizobial isolates was reduced with increasing salt tolerance in culture.
4. A large replicated glasshouse inoculation trial using 40 strains of rhizobia and nine species of *Acacia* that varied in their ability to grow in saline soils found strong evidence of host specificity, but neither the average growth promotion of host plants nor the specificity of growth promotion was related to salt tolerance of the isolates.
5. In a second glasshouse experiment, we used a subset of salt-sensitive and salt-tolerant rhizobia and six *Acacia* species to evaluate performance and symbiotic effectiveness across different levels of soil salinity. Overall, we found no evidence of a relationship between rhizobial salt-tolerance (as measured in the laboratory) and impact on host growth performance, either in saline or non-saline soils, and there was no evidence that salt-tolerant rhizobia perform better in more saline environments.
6. *Synthesis.* More salt tolerant *Acacia* spp. were less responsive in growth to rhizobial inoculation than salt sensitive hosts, implying that evolution towards reduced dependence on the symbiosis may facilitate adaptation to salt, raising a general question regarding the extent to which shifts in dependence on microbial symbionts underlies plant adaptation to other environmental gradients.

Key-words: co-adaptation, costs, host specificity, legume, mutualism, salt-tolerance, symbiosis

Introduction

In recent years, there has been increasing recognition of the need for a broader perspective for plant ecology that incorporates interactions with soil microbial communities (Reynolds *et al.* 2003; Schimel *et al.* 2007). A range of studies have shown that plant–soil interactions and the dynamic feedbacks these interactions generate, can be important organizing forces of community structure and function through both direct and indirect pathways (Klironomos 2002; Bever

2002a,b, 2003). At the same time, plant and soil communities are themselves directly influenced by environmental factors such as soil pH and salinity levels, soil toxicity and water availability. Particularly for associations between plants and symbiotic mutualists, quantifying how variation in environmental factors influences the effectiveness of the interaction is central to understanding the dynamics of community assembly (Thrall *et al.* 2007a), as well as how these associations co-evolve across geographic ranges (Parker 1999; Thompson 2005). In this context, the presence of trade-offs in different life-history components (e.g. N₂-fixing effectiveness vs. growth rate or tolerance of hostile soil factors) could strongly determine the ecological outcomes of evolutionary processes.

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There are several potential mechanisms through which evolutionary shifts in host plant interactions with soil symbionts could mediate plant adaptation to productivity or stress. First, it is possible that plant dependence on symbionts is unchanged across an environmental gradient, but that the micro-organisms themselves are sensitive to this gradient. In this case, microbial adaptation to the environmental stressor is a prerequisite for plant success and the presence of trade-offs in different microbial life-history components (e.g. growth rate vs. tolerance of hostile soil factors) would underlie the microbial evolutionary dynamic. There is evidence of such trade-offs and of the resulting adaptation of soil micro-organisms to stress (Schimel *et al.* 2007). For soil symbionts, there is also some empirical support for this adaptation resulting in improved plant performance in more stressful environments. For example, Toler *et al.* (2005) showed that *Sorghum bicolor* performed better in the presence of heavy metals in association with metal tolerant strains of mycorrhizal fungi, and Stahl & Smith (1984) found that *Agropyron smithii* exhibited greater drought tolerance when grown with mycorrhizal fungi isolated from arid environments compared to isolates from more mesic environments in the western United States.

Similarly, there is at least some evidence that the ability of legume hosts to grow and survive in saline conditions is improved when they are inoculated with salt tolerant strains of rhizobia (Zou *et al.* 1995; Hashem *et al.* 1998; Shamseldin & Werner 2005). In contrast, Lal & Khanna (1994) found no evidence of a benefit of rhizobial salt tolerance for plant performance. Moreover, it has been shown in some cases that microbial evolution in response to environmental gradients can lead to a decrease in mutualistic benefits (Corkidi *et al.* 2002; Kiers *et al.* 2002) and there is theoretical support for this (Thrall *et al.* 2007a). Overall, given conflicting results and the relatively limited number of symbiont strains evaluated in most of these studies, these examples must be regarded as illustrative, leaving the generality of these findings still undetermined.

It is also possible that plant dependence on soil symbionts might shift along an environmental gradient (e.g. in more stressful environments, hosts might evolve reduced dependence on mutualists because of associated costs or trade-offs relevant to other aspects of persistence). Plants, for example, are known to vary in dependence on mycorrhizal fungi and this variation is thought to relate to the ecology of individual plant species. Plants with low dependence on mycorrhizal fungi tend to dominate in areas with low densities of mycorrhizal fungi or where soils contain high nutrient levels (e.g. Medve 1984). Consistent with this mechanism, Schultz *et al.* (2001) found that ecotypes of big bluestem (*Andropogon gerardii* Vitman) from fertile prairies are not dependent on mycorrhizal fungi, while ecotypes of big bluestem from infertile prairies are highly dependent.

A third possibility is that adaptation and persistence along an environmental gradient requires genetic change in both the plant and symbiont. In this case, the specificity of the interaction may result in plant genotypes doing best with the symbiont genotypes from their same environment (i.e.

co-adaptation). Necessary elements of this process are well-established, including genetic changes along environmental gradients in relation to the symbiosis in the host (Schultz *et al.* 2001) and symbiont populations (Ibekwe *et al.* 1997; Delorme *et al.* 2003). There is also abundant evidence of specificity in host response to symbionts (Burdon *et al.* 1999; Thrall *et al.* 2000; Bever 2002a; Klironomos 2003) and in symbiont response to hosts (Bever 2002b). Tests in homogeneous environments have demonstrated negative feedback, which deteriorate mutualisms rather than lead to co-adaptation (Bever 2002a; Castelli & Casper 2003). However, evolution along an environmental gradient may be more likely to yield positive feedback that would generate co-adaptation (Bever 1999). Co-adaptation of host–symbiont associations along environmental gradients has yet to be conclusively demonstrated, in that (as far as we are aware) there are no studies which provide evidence that matching of adapted plant and symbiont populations results in synergistic advantages in performance.

Soil salinity is one example of an environmental stress which has become an issue of major ecological and economic importance in many parts of the world. For example, in Australia the extensive clearing of deep-rooted native perennial vegetation has resulted in significant increases in dryland salinity (National Land & Water Audit 2001). Given the prevalence and diversity of native shrubby legumes (e.g. *Acacia* spp.) in many Australian ecosystems (Groves 1994) as well as the nutrient-poor status of many of these soils, the association between these plants and N₂-fixing rhizobial bacteria represents a model system for investigating ecological and evolutionary interactions along an environmental gradient such as soil salinity, and exploring how co-evolutionary shifts in these interactions might influence community structure and function.

Previous studies of *Acacia*–rhizobial associations have shown variation in abundance and diversity (Lafay & Burdon 1998, 2001), host specificity (Burdon *et al.* 1999; Thrall *et al.* 2000), and N₂-fixing effectiveness in relation to host species, soil chemistry and physical environmental factors (Thrall *et al.* 2007b). Furthermore, it is known that both hosts (Marcar & Crawford 2004) and rhizobia (Elsheikh 1998; Zahran 1999; P.H. Thrall & L.M. Broadhurst, unpublished data) vary in salt tolerance. Of particular interest in the context of environmental stress is the identification of any underlying physiological trade-offs in key life-history features that might influence ecological and evolutionary trajectories. However, there has been little or no examination of this issue in natural plant–microbe systems, although such trade-offs could clearly impact on community composition and host–symbiont co-evolutionary dynamics.

We still lack a broad understanding of the causal links between environmental heterogeneity, population and geographic genetic variation, and ecological performance of host–symbiont associations. Improved knowledge of these interactions will not only provide insights into community and co-evolutionary dynamics, but it has applied value in the context of large-scale revegetation and ecosystem restoration of degraded agricultural landscapes (Thrall *et al.* 2005). Here we use a range of *Acacia* spp. known to vary in their ability to tolerate saline

soils and associated rhizobial symbionts to address several basic ecological questions relevant to understanding the impact of an environmental stress factor (soil salinity) on a naturally occurring symbiotic association: (i) Is there evidence for a trade-off between rhizobial growth rate and adaptation to salt stress? (ii) Is there evidence that such a trade-off could mediate bacterial N₂-fixing effectiveness and thus the ability to promote plant growth? (iii) Is there evidence that the relative performance of plant–rhizobia combinations varies along salinity gradients in relation to salt tolerance in either the host or the symbiont?

Methods

LABORATORY ASSAY OF SALT TOLERANCE

In an earlier laboratory study, we assessed the extent of variation for salt tolerance in native populations of rhizobia associated with Australian *Acacia* spp. (P.H. Thrall & L.M. Broadhurst, unpublished data). In that study, the growth and survival of 175 rhizobial strains (isolated from 17 *Acacia* host species from 22 locations across southeastern Australia) were evaluated in liquid culture at several salinity levels (0, 200, 400 and 800 mM NaCl). These strains were derived from a broad range of *Acacia* spp. native to southeastern Australia (P.H. Thrall & L.M. Broadhurst, unpublished data). Molecular studies indicate that the majority of these strains belong to either *Rhizobium* or *Bradyrhizobium* (P.H. Thrall & L.M. Broadhurst, unpublished data). Here, we re-analyse these data specifically to evaluate the possibility of a trade-off in rhizobial growth vs. salt-tolerance. We then use the results of this analysis to interpret host growth responses in relation to different levels of soil salinity in the glasshouse.

For each salinity treatment, the growth (i.e. changes in turbidity reflecting both cell densities and potential production of exopolysaccharides) of three replicates of each isolate were measured daily for 3 days. Initially bacteria were grown and stored at 28 °C on slants of yeast-extract mannitol agar (YMA). Isolates were sub-cultured and grown on slants of YMA for a minimum of 1 week, depending on bacterial vigour. Transfers from slants were used to inoculate 125 mL Erlenmeyer flasks containing 50 mL of yeast extract mannitol broth. Flasks of broth were incubated at 120 r.p.m. and 28 °C in a Thermoline TSIR-406-25 orbital shaking incubator. Isolate growth was measured spectrophotometrically at 660 nm using a Beckman DU-50 spectrophotometer to assess turbidity. Growth rates were calculated as the difference in OD₆₆₀ readings at 0 and 24 h, as the growth of many strains were observed to slow after 24 h. Responses to salinity (salt tolerance) were assessed as the ratio determined by the difference between 1 day and initial OD₆₆₀ readings for the 200 mM salt treatment divided by the difference in 1 day, and initial OD₆₆₀ readings for the no salt controls [i.e. tolerance = (OD_{200 mM,24 h} – OD_{200 mM,initial})/(OD_{0 mM,24 h} – OD_{0 mM,initial})]. This index of salt response was used in all analyses involving salt tolerance.

ASSAY OF ACACIA RESPONSE TO RHIZOBIAL ISOLATES

To assess variation in host specificity as well as rhizobial effectiveness at promoting plant growth in relation to salt tolerance, a subset of 40 of the 175 strains (Fig. 1) from the laboratory assessment of salt tolerance were used in a replicated glasshouse inoculation trial

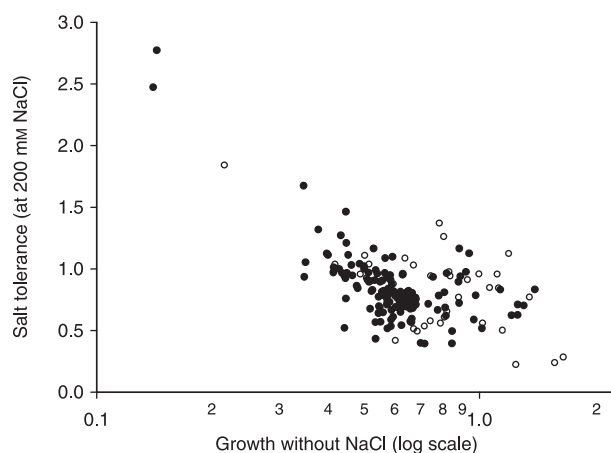


Fig. 1. Trade-off between laboratory growth rate and tolerance to salt across a range of rhizobial isolates derived from different native Australian *Acacia* species. Growth rates were measured in the laboratory in liquid culture. Salt tolerance was measured as the ratio of growth rates at 200 mM NaCl over growth in the no salt control (see Methods for detail). The open circles represent the subset of strains subsequently used to assess rhizobial effectiveness (glasshouse experiment I).

with nine *Acacia* host species. The rhizobial strains were chosen to represent the observed range of variation in growth and tolerance seen in culture (but otherwise haphazardly). The host species chosen were: *A. brachybotrya*, *A. hakeoides*, *A. ligulata*, *A. mearnsii*, *A. pendula*, *A. pycnantha*, *A. rigens*, *A. salicina* and *A. stenophylla*. These were selected to represent a broad range of geographic distributions and ecologies. In addition, these species vary considerably in salt tolerance, with *A. mearnsii* and *A. brachybotrya* among the most salt sensitive, and *A. salicina* and *A. stenophylla* among the most salt tolerant [*a priori* classification based on: (i) independent assessment by N. Marcar, pers. comm.; and (ii) review of existing literature].

For the glasshouse trial (established in July 2005), rhizobial strains were freshly grown on YMA plates. Using a wire inoculation loop, each strain was resuspended in 1.0-L YMB then placed in a shaking incubator at 27 °C for 1 week. Viability counts were then performed on each culture to ensure that similar numbers of rhizobia ($1\text{--}5 \times 10^9$ cells mL⁻¹) were used to inoculate each seedling (data not shown).

Each host species × rhizobial treatment was replicated 10 times in a completely randomized block design (= 3780 pots across 20 benches including N⁻ controls where plants were left uninoculated). Eighty millimetre diameter plastic pots were filled with sterilized vermiculite:sand (50 : 50) mixture. *Acacia* seeds were obtained from the Australian Seed Company (Hazelbrook, NSW). Seeds of *A. ligulata*, *A. mearnsii*, *A. rigens* and *A. salicina* were pre-treated with boiling water for 1 min, allowed to cool and left to stand for 24 h, while *A. brachybotrya*, *A. hakeoides*, *A. pendula*, *A. pycnantha* and *A. stenophylla* seed was individually nicked. After pre-treatment, seed was transferred to germination trays and watered daily. Emerging seedlings were planted into pots after 7–14 days. Three days after planting, each seedling was inoculated with 5 mL of the appropriate inoculant (a relatively large volume of inoculant was used to minimize potential cross-contamination issues). Pots were covered with polyethylene beads to eliminate splashing and cross contamination between adjacent pots. Plants were grown under standard glasshouse conditions (16 h at 25 °C; 8 h at 18 °C), watered as needed with UV-sterilized tap water and weekly with N-free McKnight's solution.

Plants were harvested after 14 weeks and above-ground biomass was oven dried (65 °C, 48 h) and weighed separately for each plant. Dry weights were used as a measure of symbiotic effectiveness. At harvest, each plant was also assessed for: (i) nodule number (0, 1–10, 10–50, > 50), (ii) nodule functionality, based on colour and size [scores ranged from 1 (small non-N₂-fixing nodules with white centres) to 5 (large nodules with pink/red centres)], and (iii) nodule distribution (scores ranged from 1 to 5, with low scores representing plants with nodules distributed all or mostly within the root crown, and high scores denoting plants with nodules more broadly distributed throughout the root system).

A subset of the control plants formed nodules late in the experiment; these were generally small and few in number (average < 10), and only occurred on lateral roots. This resulted in negligible impact on plant performance as evidenced by consistently low values for above-ground biomass in this group (Table S1 in Supplementary Material). Moreover, removal of contaminated controls from the analyses did not alter any of our interpretations.

TEST OF RHIZOBIAL MEDIATION OF ACACIA ADAPTATION TO SOIL SALINITY

Three salt tolerant strains [CPI257 (host of origin: *A. implexa*), CPI237 (*A. nanodealbata*) and CPI241 (*A. binervata*)] and three salt sensitive strains [CPI234 (*A. dealbata*), CPI258 (*A. trachyphloia*) and CPI238 (*A. deanei*)] were selected on the basis of their similar average levels of effectiveness across the nine *Acacia* species used in the effectiveness trial. Salt tolerance was based on the results from the laboratory cultural study. The three salt tolerant and three salt sensitive strains were grown separately in 500 mL YMB in a shaking incubator for 1 week. For the salt tolerant treatment, individual broths containing strains CPI257, CPI237 and CPI241 were combined into a single bulk inoculant. Similarly, the salt sensitive treatment contained equal volumes of strains CPI234, CPI258 and CPI238.

To examine variation in rhizobial effectiveness and host performance in relation to soil salinity, six salt concentrations were used (0, 50, 100, 200, 400 and 800 mM NaCl, the primary form of soil salinity across the Murray Darling Basin; National Land & Water Audit 2001) across four rhizobial treatments (salt tolerant strains, salt sensitive strains and uninoculated N⁺ and N⁻ controls) in factorial combination with six *Acacia* host species (*A. brachybotrya*, *A. ligulata*, *A. mearnsii*, *A. pycnantha*, *A. salicina* and *A. stenophylla*) that spanned the range of ability to tolerate saline conditions. Each host species × rhizobial treatment × salt treatment was replicated 10 times in a split plot design to facilitate the complex watering arrangements. The experiment was established in November 2006.

As for the first glasshouse experiment, 80 mm diameter plastic pots were filled with a 50 : 50 sterilized vermiculite:sand mixture. Seeds of *A. ligulata* and *A. mearnsii* were pre-treated with boiling water, *A. brachybotrya* and *A. pycnantha* seed were individually nicked, and *A. salicina* and *A. stenophylla* seed pre-treated with sulphuric acid for 45 min followed by repeated rinsing with distilled water. Procedures for the planting of pre-treated seed and young seedlings followed those used in the glasshouse effectiveness study. Seedlings were then inoculated 4–6 days after planting with 5 mL of either the salt tolerant or salt sensitive bulk inoculant and the soil surface was covered with polyethylene beads.

Glasshouse conditions were the same as those described for the effectiveness study. Prior to initiation of the salt treatments, plants were watered with UV-sterilized tap water. Salinity treatments were initiated 2 days after inoculation to allow sufficient time for symbiosis to occur. For each treatment, the salt was added to a 10% McKnight's

solution. To avoid salinity shock, salt concentrations were increased by 50 mM NaCl per day until the final experimental concentrations were reached (e.g. for the 800 mM NaCl treatment, this was 16 days after salt was first applied). Once each salt concentration was reached, soil moisture was maintained for the duration of the experiment using the appropriate NaCl concentration (0, 50, 100, 200, 400 or 800 mM NaCl) in 15% McKnight's solution. Nitrogen was applied weekly to each N⁺ control at a rate of 10 mL of 0.5% KNO₃.

Plant survival was monitored on a weekly basis, and plants dying before the end of the experiment at 16 weeks were harvested at that time. At 16 weeks, the experiment was terminated and all remaining plants harvested. Above-ground biomass was oven dried at 60 °C for 48 h and each plant weighed separately. Plant roots were separated and nodulation characteristics recorded as for the glasshouse effectiveness trial.

To determine the actual levels of soil salinity reached in each salt treatment, a separate set of pots were planted with individual seedlings of *A. stenophylla*, and appropriate salt treatments applied (as described above). For each salt treatment, three pots were removed on a weekly basis (up to 16 weeks), and substrate electrical conductivity was measured using a handheld conductivity meter (TPS model WP-81).

STATISTICAL ANALYSIS

Laboratory assessment of salt tolerance

We note that if the growth of all isolates were decreased by the same proportion [i.e. growth with salt = $a \times$ (growth without salt), where $a < 1$], then our measure of salt tolerance should be uncorrelated with growth in low salt [i.e. salt tolerance = (growth in salt)/(growth without salt) = a]. If, however, there is a cost of salt tolerance that is manifest as reduced growth in the absence of salt, this cost would generate a negative relationship between salt tolerance and growth in the absence of salt, thus providing evidence of a trade-off. The Pearson product-moment correlation between salt tolerance and isolate growth without salt was measured using SAS PROC CORR (SAS Institute Inc., Cary NC). Growth rates in the no salt control were log-transformed to improve the fit to parametric assumptions.

Rhizobial effectiveness (glasshouse experiment I)

The relationship between rhizobial salt tolerance (as assessed in the laboratory), and effectiveness at promoting growth of *Acacia* host plants (plant dry weights) in the glasshouse was tested in two ways. First, we tested the correlation coefficients of mean growth promotion of *Acacia* spp. in relation to laboratory measures of rhizobial salt tolerance and growth in culture. Second, we included the laboratory measures of salt tolerance of the rhizobial isolates as well as interactions between this variable and host species as predictors of host growth within a mixed model ANOVA. The rhizobial isolates and their interaction with host species was used as a random effect. This ANOVA was performed using SAS PROC MIXED (SAS Institute Inc.).

Rhizobial mediation of host salt tolerance (glasshouse experiment II)

The test of co-adaptation (i.e. the expectation that matching salt tolerant acacias and salt tolerant rhizobia would produce synergistic advantages in performance) was analyzed using a mixed model ANOVA with block and the interaction of block with salt treatment and their interactions being treated as random effects (as appropriate

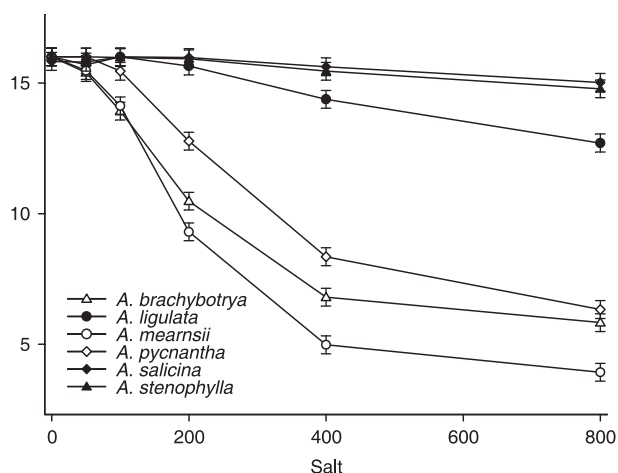


Fig. 2. Variation among *Acacia* species with regard to ability to tolerate saline conditions [note that for calculations of slope used in subsequent analyses only the first five data points were used for the most salt-sensitive species (*A. mearnsii*, *A. brachybotrya*, *A. pycnantha*) as survival for these species did not further decline above 400 mm NaCl].

given the nested design). Inoculation treatment and all interactions with inoculation were split into three contrasts that corresponded to the effect of N addition, the average effect of rhizobial inoculation and the differential effect of the two rhizobial inocula (salt-sensitive vs. salt-tolerant). We first analyzed plant survival using the entire data set. Because of the high mortality in the high salt treatments (particularly 400 and 800 mm), we were unable to analyze plant growth for the full data set. Our analysis of plant growth was therefore limited to the treatments receiving 200 mm NaCl or less. We used the same subset of data to test for differences in nodule number and nodule distribution.

To look for patterns in acacia response to N and rhizobia, and the salt tolerance of *Acacia* spp., we constructed a measure of acacia salt tolerance as the slope of number of weeks survived against salt concentration (Fig. 2). As this relationship was strongly nonlinear for the most salt sensitive acacia species (*A. mearnsii*, *A. brachybotrya*, *A. pycnantha*; Fig. 2), we only used the data for salt concentrations of 400 mm and less for these species. A *t*-test was used to test the correlations between salt tolerance and growth response of the different *Acacia* spp.

Results

TESTS OF COSTS AND CORRELATIONS OF SALT TOLERANCE IN RHIZOBIA

There was a strong negative correlation between salt tolerance and laboratory growth rate in the absence of salt ($r = -0.50$, $n = 175$, $P < 0.0001$; Fig. 1). However, there was no relationship between salt tolerance or growth rate observed in the laboratory and the average growth promotion of acacias in the glasshouse effectiveness trial ($r = 0.16$, $n = 40$, NS).

ANALYSIS OF ACACIA GROWTH RESPONSES TO RHIZOBIAL ISOLATES

The 40 rhizobial isolates used in the effectiveness trial (glasshouse experiment I) varied considerably in both their

Table 1. Mixed model ANOVA of the number of weeks survived in the co-adaptation test. Salt and all of the interactions with salt were treated as random effects

Effect	d.f.	F value	P value
Host species	5, 270	203	< 0.0001
Inoculation	3, 162	43.2	< 0.0001
N-addition	1, 162	117	< 0.0001
Average of rhizobia	1, 162	11.8	0.0007
Among rhizobia	1, 162	0.31	NS
Host species × inoculation	15, 809	7.41	< 0.0001
Host sp. × N-addition	5, 809	16.4	< 0.0001
Host sp. × average rhizobia	5, 809	5.53	< 0.0001
Host sp. × among rhizobia	5, 809	1.05	NS
Salt	5, 45	254.74	< 0.0001
Salt × host species	25, 270	34.3	< 0.0001
Salt × inoculation	15, 162	4.94	< 0.0001
Salt × N-addition	5, 162	12.94	< 0.0001
Salt × average rhizobia	5, 162	1.50	NS
Salt × among rhizobia	5, 162	0.13	NS
Salt × host sp. × inoculation	75, 809	1.83	< 0.0001
Salt × host sp. × N-addition	25, 809	3.60	< 0.0001
Salt × host sp. × average rhizobia	25, 809	1.60	0.03
Salt × host sp. × among rhizobia	25, 809	0.74	NS

average ability to promote growth of acacia host plants (covariance estimate = 0.061, SE = 0.015, $P < 0.0001$) as well as in the specificity of their growth promotion across the nine *Acacia* host species (covariance estimate = 0.059, SE = 0.005, $P < 0.0001$; see Table S1). The salt tolerance of individual rhizobial isolates (as determined in the laboratory cultural experiment) was not a significant predictor of average isolate effectiveness or of the specificity of host response.

ANALYSIS OF HOST SURVIVAL IN THE CO-ADAPTATION TEST

There were significant differences among *Acacia* spp. in their sensitivity to salt (Fig. 2; Table S2), as well as in their responsiveness to nitrogen addition (N^+ controls) and rhizobial inoculation as reflected in the significant interactions between host species, salt and inoculation (Table 1). Interestingly, salt-tolerance of plant species (in the context of survival time) was negatively correlated with responsiveness to added nitrogen ($r = -0.93$, $P < 0.01$, Fig. 3a). However, there was not a consistent relationship between plant tolerance of salinity and the responsiveness of the species to rhizobial inoculation (Fig. 3b). The significant three-way interaction between host species, inoculation treatment and salinity was a consequence of the fact that all plant species had high survivorship without salt and the differences between the plant species, as well as the sensitivity of their response to inoculation, increased with salinity.

There were no significant differences between the salt-sensitive and salt-tolerant inocula with regard to either the average survivorship of the different *Acacia* hosts, or with respect to differential survivorship of host species across different salt treatments.

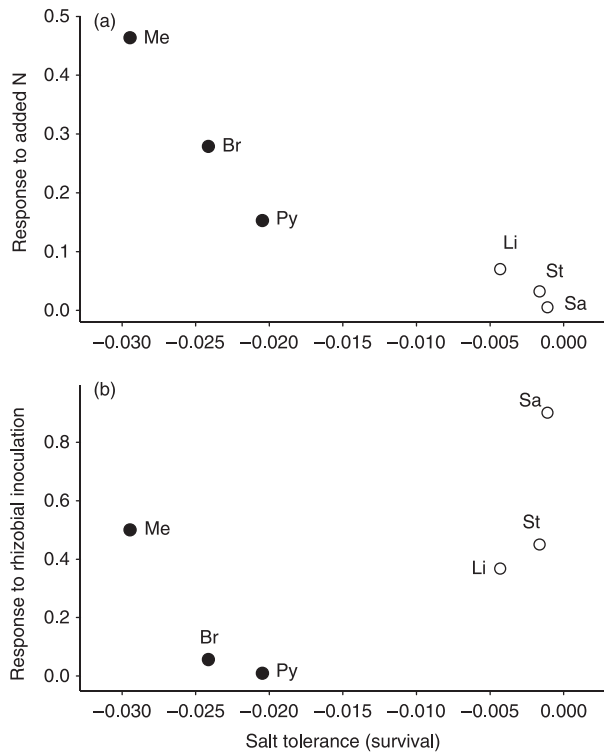


Fig. 3. Relationship between salt tolerance and responsiveness to N addition vs. rhizobial inoculation in terms of plant survivorship (number of weeks in the glasshouse co-adaptation trial). (a) Relativized survival responses to N [calculated as: (survival with N – survival without N)/survival without N]; (b) Survival in response to rhizobial inoculation [calculated as: (survival with rhizobia – survival without rhizobia)/(survival with N – survival without N)]. Salt tolerance was measured as the regression coefficient of the average number of weeks survived against salinity. Species codes are: Me, *A. mearnsii*; Br, *A. brachybotrya*; Py, *A. pycnantha*; Li, *A. ligulata*; Sa, *A. salicina*; St, *A. stenophylla*. Salt-sensitive and salt-tolerant host species are represented by filled and open circles, respectively.

ANALYSIS OF HOST GROWTH IN THE CO-ADAPTATION TEST

Plant species again varied in the extent to which their growth was inhibited by salinity. Tolerance to salinity in growth (as measured by the slope of average growth with increasing salinity) was marginally correlated to the tolerance to salinity observed in the survivorship data ($r = 0.74$, $P = 0.06$). *Acacia* species also varied in their growth response to added nitrogen vs. inoculation with rhizobia (contrasts in Table 2). There was no correlation between the tolerance to salinity of the plant species and their responsiveness to nitrogen addition (Fig. 4a). However, there was a negative correlation between the tolerance of plant species to salinity and their responsiveness to rhizobial inoculation ($r = -0.82$, $P < 0.05$, Fig. 4b).

The average growth responsiveness of the acacias to N and to inoculation to rhizobia depended upon salinity (Table 2). The average growth responsiveness to N increased linearly with salinity ($r = 1.00$, $P < 0.01$, Fig. 5), while the average responsiveness to rhizobia declined with salinity ($r = -0.98$, $P < 0.01$, Fig. 5). There was a corresponding decline in the

Table 2. Mixed model ANOVA of log plant dry weight in the co-adaptation test. Salt and all of the interactions with salt were treated as random effects

Effect	d.f.	F value	P value
Host species	5, 180	35.7	< 0.0001
Inoculation	3, 108	112	< 0.0001
N-addition	1, 108	304	< 0.0001
Average of rhizobia	1, 108	32.2	< 0.0001
Among rhizobia	1, 108	1.6	NS
Host species × inoculation	15, 539	7.21	< 0.0001
Host sp. × N-addition	5, 539	6.56	< 0.0001
Host sp. × average rhizobia	5, 539	9.39	< 0.0001
Host sp. × among rhizobia	5, 539	0.91	NS
Salt	3, 27	147	< 0.0001
Salt × host species	15, 180	5.97	< 0.0001
Salt × inoculation	9, 108	1.48	0.16
Salt × N-addition	3, 108	2.3	0.08
Salt × average rhizobia	3, 108	3.28	0.02
Salt × among rhizobia	3, 108	0.41	NS
Salt × host sp. × inoculation	45, 539	1.32	0.08
Salt × host sp. × N-addition	15, 539	1.46	0.116
Salt × host sp. × average rhizobia	15, 539	1.82	0.03
Salt × host sp. × among rhizobia	15, 539	1.07	NS

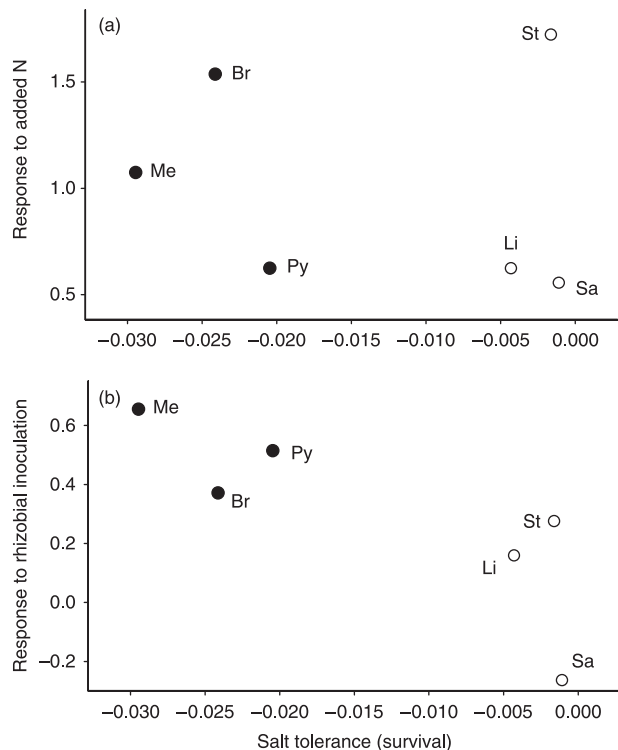


Fig. 4. Relationship between salt tolerance and growth responsiveness to N addition vs. rhizobial inoculation. (a) Relativized growth responses to N [calculated as: (growth with N – growth without N)/growth without N]; (b) Growth responses to rhizobial inoculation [calculated as: (growth with rhizobia – growth without rhizobia)/(growth with N – growth without N)]. Tolerance to salinity was measured as the regression coefficient of average plant size (g dry weight) against salinity. Species codes are: Me, *A. mearnsii*; Br, *A. brachybotrya*; Py, *A. pycnantha*; Li, *A. ligulata*; Sa, *A. salicina*; St, *A. stenophylla*. Salt-sensitive and salt-tolerant host species are represented by filled and open circles, respectively.

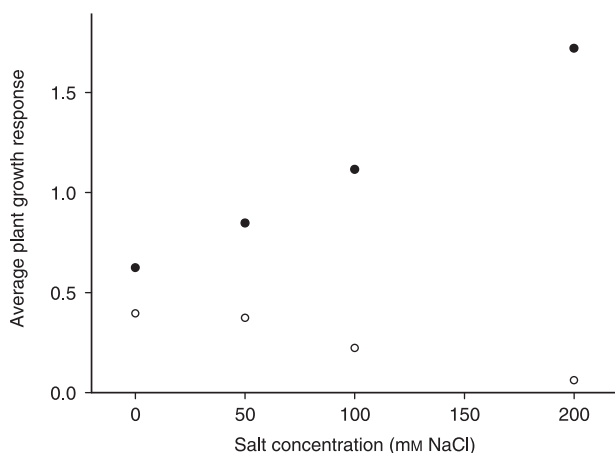


Fig. 5. Average plant growth (across all *Acacia* spp.) in response to nitrogen addition and rhizobial inoculation for different levels of soil salinity. Solid circles = response to N, open circles = response to rhizobial inoculation relativized by response to N. Relativized response data were calculated as described for Fig. 4.

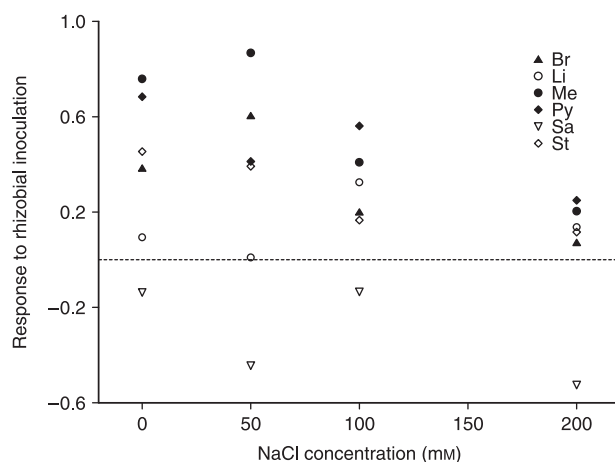


Fig. 6. Growth response of individual *Acacia* spp. to rhizobial inoculation in relation to different levels of soil salinity. Relativized growth responses were calculated as described for Fig. 4b. Species codes are: Me, *A. mearnsii*; Br, *A. brachybotrya*; Py, *A. pycnantha*; Li, *A. ligulata*; Sa, *A. salicina*; St, *A. stenophylla*. Salt-sensitive and salt-tolerant host species are represented by filled and open circles, respectively.

number and distribution of nodules on plant roots with increasing salt ($F_{3,27} = 10.1$, $P < 0.0001$, $F_{3,27} = 13.3$, $P < 0.0001$, for nodule number and nodule distribution, respectively). Individual species of *Acacia* differed in the rate of decline of rhizobial response to salinity (significant three-way interaction contrast in Table 2). In general, the salt-sensitive *Acacia* species experienced a greater decline in rhizobial response with salinity than the salt-tolerant *Acacia* species (Fig. 6).

There were no significant differences in the growth response of acacias to the two rhizobial inocula. However, there was a significant interaction between rhizobial source and host species for nodule number and nodule distribution ($F_{5,179} = 3.10$, $P = 0.01$; $F_{5,180} = 2.88$, $P < 0.02$, for nodule

number and nodule distribution, respectively). This appeared to be largely due to *A. salicina* having a greater number of nodules and better nodule distribution with the salt-tolerant strains, while *A. stenophylla* had greater nodule numbers and better nodule distribution with the salt-sensitive strains.

Discussion

This study affirms several basic tenets of microbial mediation of plant adaptation to salinity stress. First, we found a strong trade-off between tolerance to salt and growth rate in the absence of salt in rhizobia isolates. However, salt-tolerant strains of rhizobia did not differ from salt-sensitive strains in their ability to promote growth of a range of *Acacia* spp. even in saline soils. Moreover, while we did find strong evidence of host-specificity in the response of different *Acacia* spp. to particular rhizobial strains, we did not find that this specificity of response was aligned as expected under the hypothesis of co-adaptation. Overall, there was evidence of rhizobial mediation of *Acacia* adaptation to salinity, as we observed differences in salinity tolerance among *Acacia* spp. and salt-tolerant acacias differed from salt-sensitive species in their interactions with rhizobia, in that salt-tolerant acacias had reduced dependence on rhizobia. This reduced dependence could be a critical aspect of ecological success in highly saline conditions. We discuss the significance of each of these results in turn below.

First, our results show that strains of rhizobial bacteria with high tolerance to salinity also have reduced growth rates in the absence of salinity, as would be expected from a cost of tolerance to salinity (and has been shown more generally for a number of other environmental stress factors; Schimel *et al.* 2007). This result contrasts with the frequent conclusion that rhizobial growth rate and salt-tolerance are positively correlated (e.g. Elsheikh 1998; Marsudi *et al.* 1999; Barboza *et al.* 2000; Zerhari *et al.* 2000), although this difference may be attributable to the fact that most previous studies did not factor out intrinsic variation in growth rates from analyses of salt-tolerance. From a community perspective, the trade-off that we demonstrate suggests that salt-tolerant strains will be replaced by faster-growing salt-sensitive strains in soils of low salinity. That is, as a result of this cost, we expect to find genetic and species differentiation of rhizobial communities along a salinity gradient. Although not conclusive, evidence from other work on rhizobial salt tolerance (P.H. Thrall & L.M. Broadhurst, unpublished data) is consistent with this idea. In that study, RFLP analysis of strains from both non-saline and saline soils found that the majority of rhizobial genomic species present in saline soils were not present in the non-saline sites. Other studies have suggested that the relative predominance of different rhizobial genera may shift in relation to environmental stress (e.g. Barnett & Catt 1991; Jenkins 2003). More generally, soil microbial abundance, community structure and functional representation by different microbial groups have also been shown to respond to gradients in soil salinity (Omar *et al.* 1994; Nelson & Mele 2007; Schimel *et al.* 2007).

Clearly, shifts in symbiont community composition along environmental gradients could have consequences for the relative ecological success of different host species. Such shifts could also influence the co-evolutionary dynamics of host–symbiont associations, particularly where host specificity and symbiotic effectiveness are factors. In the case of *Acacia*–rhizobial interactions, previous studies have indeed shown considerable variation among host species in their responses to particular rhizobial isolates (Burdon *et al.* 1999; Thrall *et al.* 2000; Murray *et al.* 2001). We also found substantial variation in average growth promotion of rhizobial isolates and in the specificity of this growth promotion in the glasshouse. Depending on how rhizobial community composition responds to increasing salinity, and further how trade-offs in growth and tolerance relate to the levels of mutualistic benefits conferred, this could alter the relative competitive abilities of salt-sensitive vs. more salt-tolerant acacias along salinity gradients. In the context of co-adaptation, this could also lead to distinctly different co-evolutionary dynamics in different soils.

However, our current results do not provide evidence for this specificity of rhizobial growth promotion playing an important role in acacia growth in saline soils. In fact, we did not observe any differences in average growth promotion or specificity of growth promotion in a survey of 40 rhizobial strains chosen from across the salt-tolerance spectrum identified in the laboratory cultural experiments. Similarly, we did not detect any difference in growth promotion between the mixed salt tolerant inoculum and the mixed salt sensitive inoculum in the direct test of co-adaptation (glasshouse experiment II). Several previous studies, largely based on comparison of single pairs of rhizobial strains varying in salt-tolerance, have either shown that inoculation with salt-tolerant strains of bacteria may reduce the adverse impacts of salt relative to salt-sensitive rhizobia (Zou *et al.* 1995; Hashem *et al.* 1998; Shamseldin & Werner 2005), or found no added benefit of rhizobial salt-tolerance for plant performance in saline soils (Lal & Khanna 1994). Therefore, at present there is no consistent evidence that rhizobial adaptation to salinity generally benefits plant growth – the present study is perhaps the most comprehensive evaluation of variation in salt-tolerance in native systems to date.

Interestingly, we found that salt-tolerant species of *Acacia* had reduced responsiveness to rhizobia, both in survival and growth (the former may be an artefact, given that a positive response to N was constrained by high survival in the N-free control). In fact, *A. salicina*, which, along with *A. stenophylla*, exhibited very high levels of tolerance to salt (Fig. 2), did not benefit from rhizobial inoculation in any salt concentration (Fig. 6). Moreover, hosts with low salt-tolerance, such as *A. mearnsii*, had high responsiveness to rhizobial inoculation which was sharply reduced with increasing salinity. The reduced dependence of salt-tolerant acacias on rhizobia could be an adaptive response to several physiological and ecological forces (Fig. 7).

First, there could be a reduction in the physiological efficiency of symbiotic N₂-fixation associated with saline soils (identified as causal pathway C in Fig. 7). Consistent with

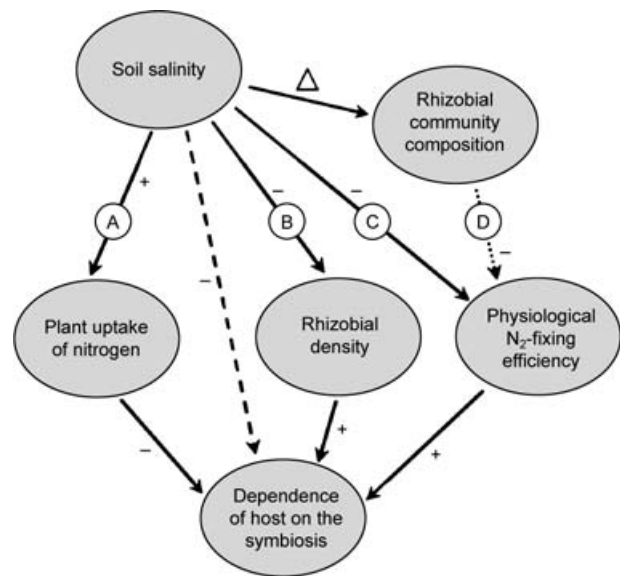


Fig. 7. Conceptual diagram illustrating hypotheses regarding the relationships between soil salinity, rhizobial community composition and abundance, and impacts on the evolution of the plant–rhizobial interaction towards lower host dependence on the symbiosis in more saline environments (Δ = non-directional change). This could either be an indirect consequence of reduced rhizobial density (path B), a response to reduced physiological benefits from the association (path C), or changes in plant uptake of N (path A). Solid arrows indicate pathways for which there is empirical evidence, either from the current study or in existing literature, while dotted or dashed arrows indicate hypothesized relationships. Our study demonstrates a cost to rhizobia for salt tolerance which would generate genetic differentiation along a salt gradient. Our results also indicate a general decline in plant growth promotion (N₂-fixing effectiveness) with increasing salinity, but do not support the idea that more salt-tolerant rhizobia differ in mutualistic benefits from salt-sensitive rhizobia (path D).

this, we observed that across all *Acacia* spp. the responsiveness to rhizobia decreased with increasing salinity (Fig. 4b), while the responsiveness to N increased (in terms of both survival and growth). This suggests that while N limitation is still important in saline conditions, N-fixation becomes a less effective substitute for soil N. For example, N₂-fixing activity has been shown to decline under salt stress (Zahran 1991, 1999; Lal & Khanna 1994; Hashem *et al.* 1998).

It is also possible that this reduction in efficiency is partly due to reduced rates of proliferation of rhizobial bacteria in saline conditions (causal pathway B in Fig. 7). A range of surveys have found that the overall density of rhizobia can be strongly reduced in saline soils (Singleton *et al.* 1982; Elsheikh 1998; Zahran 1999; Slattery *et al.* 2001). Impairment of the infection process by salinity has also been observed previously (Singleton *et al.* 1982; Craig *et al.* 1991; Zahran 1999) and our finding of reduced nodule density and poorer distribution of nodules with increasing salinity is consistent with this interpretation. The reduced density of rhizobial soil populations and the reduced efficiency of the association could alter the cost-benefit ratio in favour of reduced dependence as part of host adaptation to saline soils.

Another potential selective force for reduced dependence would arise if salt-tolerant rhizobia are less effective mutualists (causal pathway D in Fig. 7). This possibility is suggested by previous work (Zahran 1999), but is not supported by the present study. Finally, from an ecological perspective, cost benefit considerations of symbiotic N₂-fixation also depend upon the availability of soil N, which might actually increase in high salinity due either to reduced interspecific competition and lower plant densities, or to negative impacts of salinity on nutrient uptake rates by plants (possibly due to reduced photosynthetic rates and demand for N; Sprent & Zahran 1988). This would also select for reduced host dependence on rhizobia (causal pathway A in Fig. 7). Relatively little work has examined nutrient dynamics in natural plant communities subject to soil salinity – one study of native *Acacia* species in the Negev desert found a positive relationship between soil salinity and nitrogen (and a negative relationship between N and plant diversity; Munzergova & Ward 2002), and strong positive correlations have been found between plant N and soil salinity in at least some agroecosystems (van Groenigen & van Kessel 2002). The potentially complex interactions between soil N, microbial activity, and plant community structure and function in relation to environmental stresses such as salinity are clearly worth further investigation in the current context.

A general expectation, based on both theoretical and empirical studies, is that mutualisms should be stronger in lower quality environments (Thrall *et al.* 2007a). There is good empirical support for this idea (e.g. the relationship between nitrogen fertilization and other aspects of soil fertility and the level of benefits conferred by rhizobia and mycorrhizal fungi: Johnson 1993; Corkidi *et al.* 2002; Denison & Kiers 2004). There is also support for shifts in host dependence on mutualists along such productivity gradients, for example, big bluestem (*Andropogon gerardii*; a dominant grass in central USA), derived from infertile sites, is highly responsive to mycorrhizal fungi, whereas the responsiveness of big bluestem from fertile sites is reduced (Schultz *et al.* 2001). The results from the current study appear to contradict this idea, in that more salt-tolerant *Acacia* species (presumably found in more saline, lower quality environments) actually showed less dependency on rhizobia than salt-sensitive hosts from less stressful environments. However, much of the theory on the evolution of host–symbiont interactions (Thrall *et al.* 2007a) assumes that the productivity axes along which host–symbionts evolve are also correlated with the axes relating to the symbiosis itself (e.g. gradients in N availability and legume–rhizobial mutualisms). This will clearly not be the case for many other factors relating to environmental quality. For example, in the current study the gradient in soil salinity is not closely correlated with a gradient in the mutualistic benefit of N₂-fixation. This leaves the interaction free to evolve in other directions (e.g. reduction in host dependence in lower quality environments and possible increase in saprophytic ability/parasitism in the symbiont, rather than increased mutualism). This suggests that the evolutionary flexibility of host–symbiont systems depends on the degree of independence of the axes associated with the symbiosis vs. those associated with environmental quality.

Overall, the present study presents evidence for reduced strength of the plant–rhizobial mutualism as part of adaptation to salinity. Further work is necessary to separate the multiple causal pathways that could generate this result (Fig. 7). Given that Australian *Acacia* spp. also associate with mycorrhizal fungi (Warcup 1980), it would be of interest to examine how these might further modify plant responses to environmental stress. While shifts in nutritional mutualisms are expected along a fertility gradient, we do not know the extent to which shifts in dependence on microbial symbionts underlies plant adaptation to other environmental factors.

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Supplementary material

The following supplementary material is available for this article:

Table S1 Additional host growth data.

Table S2 Additional host survival data.

This material is available as part of the online article from:
<http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2745.2008.01381.x>

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