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## Comments

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# A microbiome engineering framework to evaluate rhizobial symbionts of legumes

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## Abstract

**Background** For well over a century, rhizobia have been recognized as effective biofertilizer options for legume crops. This has led to the widespread use of rhizobial inoculants in agricultural systems, but a recurring issue has emerged: applied rhizobia struggle to provide growth benefits to legume crops. This has largely been attributed to the presence of soil rhizobia and has been termed the ‘rhizobial competition problem.’

**Scope** Microbiome engineering has emerged as a methodology to circumvent the rhizobial competition problem by creating legume microbiomes that do not require exogenous rhizobia. However, we highlight an alternative implementation of microbiome engineering that focuses on untangling the complexities of the symbiosis that contribute to the rhizobial competition problem. We outline three approaches that use different starting inocula to test hypotheses to overcome the rhizobial competition problem.

**Conclusions** The approaches we suggest are targeted at various stages of the legume-rhizobium symbiosis and will help us uncover underlying molecular mechanisms that contribute to the rhizobial competition problem. We conclude with an integrative perspective of these different approaches and suggest a path forward for future research on legumes and their complex microbiome.

**Keywords** Elite inoculants · Host control · Legume-rhizobia · Microbiome engineering · Rhizobial competition problem · Symbiosis

## Introduction

For decades, there has been an attempt to inoculate rhizobia into the soil of legume crops to reduce or replace the use of expensive and polluting nitrogen fertilizer (Ferguson et al. 2010; Tilman et al. 2002). Rhizobia are commercially available as elite inoculants that are selected for application to agricultural fields based on their capacity to *improve legume growth and yield through nitrogen fixation* (USDA 2015). Elite inoculants are often tested under controlled settings that highlight the potential for benefit provided, but elite inoculants rarely provide the expected level of benefit to legumes in agricultural settings (Streeter 1994; Triplett and Sadowsky 1992; Yates et al. 2011). The problem is not that introduced rhizobia in the elite inoculants stop fixing nitrogen in this setting. Rather, the problem is strong competition with the existing soil rhizobia community, native or naturalized, that limits survivorship in the soil and access to host tissue. This phenomenon, whereby elite inoculants struggle to provide benefit in agricultural fields that is comparable to the benefit observed under controlled settings, has been termed the ‘rhizobial competition problem’ (RCP; Triplett and Sadowsky 1992). If we are to effectively

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address the RCP, we must identify the soil microbe community dynamics that inhibit elite inoculant benefit.

Several strategies to mitigate the RCP relate to the idea of increasing inoculum density to facilitate infection by elite rhizobia (Deaker et al. 2004; Rocha et al. 2019; Santos et al. 2019). Direct application of rhizobia to the seed, plant, or soil has been used to enhance infectability, but low rhizobia viability using this approach has proven problematic (Deaker et al. 2004; Rocha et al. 2019). Coating materials, such as peat and a variety of polysaccharides, have been used to increase the survival of the rhizobia, but it is unclear if these coatings result in more successful infection (Rocha et al. 2019). Additionally, recently developed inoculants contain multiple strains or species of rhizobia to increase the chance that one of the introduced rhizobia genotype will provide benefit to the target legume (Santos et al. 2019). While these strategies address the fact that elite rhizobia are outnumbered by soil rhizobia (Martínez-Romero 2003), *the intricacies of the legume-rhizobia symbiosis require additional approaches to overcome the RCP* (Checcucci et al. 2017, 2018; Poole et al. 2018).

## Engineering a microbiome tool to the solution

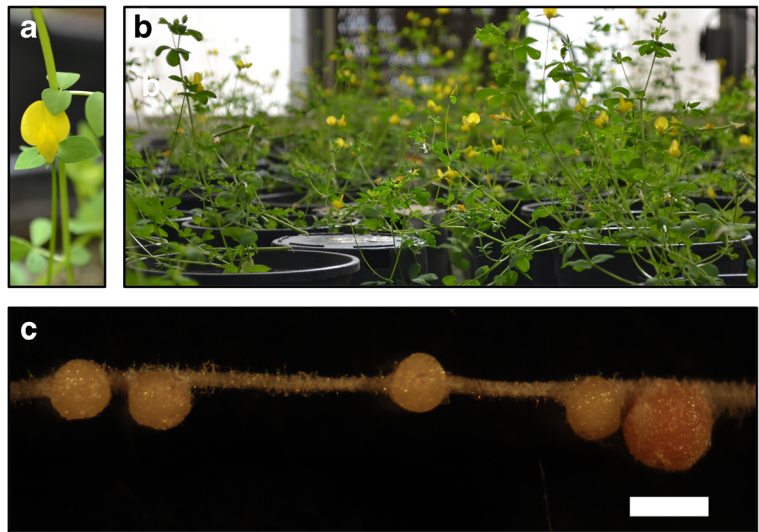
Recent developments in technology and surging interest in microbiome methodology provide a new opportunity for research in the quest for elite rhizobial inoculants. In particular, microbiome engineering has recently been proposed as a set of methods that can help us understand and assemble microbial communities (Mueller and Sachs 2015). This methodology has two focal steps. First, plant host mechanisms shape the microbial community the host interacts with. Second, a host with a trait of interest (ex., fruit production) is identified and the corresponding host microbiome is applied to new, microbe-free, hosts. By repeatedly transferring microbial communities from hosts with a trait of interest, to microbe-free hosts (i.e., passaging), the final engineered microbiome has been shown to influence artificially selected plant traits such as biomass (Swenson et al. 2000) and flowering (Pannebuisse et al. 2015). Microbiome engineering is reliant on strong host control mechanisms that shape microbial communities. Legumes, in particular, are highly amenable to this process (Fig. 1), as they have been demonstrated to dramatically alter microbial communities *in planta* and *ex planta* (Miranda-Sánchez et al. 2016; Tkacz et al. 2015; Zgadzaj et al. 2016).

Microbiome engineering has predominantly focused on assembly of beneficial microbiomes for plant hosts, but this methodology was also proposed as a way to *identify changes to the abundance of microbial species and changes to allele frequencies in microbial populations of microbiomes* (Mueller and Sachs 2015), which are both central components of the RCP that we explore. The barriers that arise due to the RCP are reflected in the four stages of rhizobia transmission: (1) root infection, (2) persistence within host tissue, (3) release from host roots, and (4) survival in the soil. Microbiome engineering, complemented by the affordability of next generation sequencing, allows us to design approaches that can rapidly identify shifts at any of these four stages of transmission. Coupling these two methodologies, we describe strategies to diagnose the RCP throughout the legume-rhizobia symbiosis. First, we provide a general outline for engineering legume microbiomes as a means of understanding the RCP. We then explore three specific approaches to further our understanding of the RCP: (1) a commercially available inoculum approach, (2) a hyper-competitive inoculum approach, and (3) a synthetic community approach.

## General microbiome engineering

The RCP involves four stages of rhizobia transmission (Fig. 2), and the selection pressures experienced at each stage are all incorporated into microbiome engineering. Just prior to infection, legumes and rhizobia engage in a ‘molecular dialogue’ that limits infection by rhizobia that provide little to no benefit (Liu and Murray 2016). However, some minimally beneficial rhizobia can still produce a compatible molecular signal despite deficiencies in nitrogen fixation (Burghardt 2019; Sachs et al. 2018). Once inside host tissue, ongoing communication with the host via polysaccharides allows rhizobia to persist within host tissue (Via et al. 2015) and fix atmospheric nitrogen (Coba de la Peña et al. 2018). Many rhizobia persist without fixing atmospheric nitrogen, and several legume hosts have demonstrated mechanisms that limit proliferation of rhizobia that provide minimal fixed nitrogen (Sachs et al. 2018). As the interaction nears an end, a subset of viable rhizobia are released from nodules back into the soil (Puppo et al. 2004). The released rhizobia are a culmination of rhizobia infection and persistence that have been shaped by legume and rhizobia traits. The released rhizobia then

**Fig. 1** *Lotus japonicus* is a commonly used legume to study the legume-rhizobia symbiosis. **a** An eight-week-old flowering plant. **b** Several 10 weeks old plants grown in 4-liter pots in a greenhouse. **c** Four-week-old nodules. The pink coloring of the far-right nodule is caused by leghemoglobin, an oxygen carrier vital to the nitrogen fixation process. Scale bar = 1 mm. Photo credit: Kenjiro W. Quides



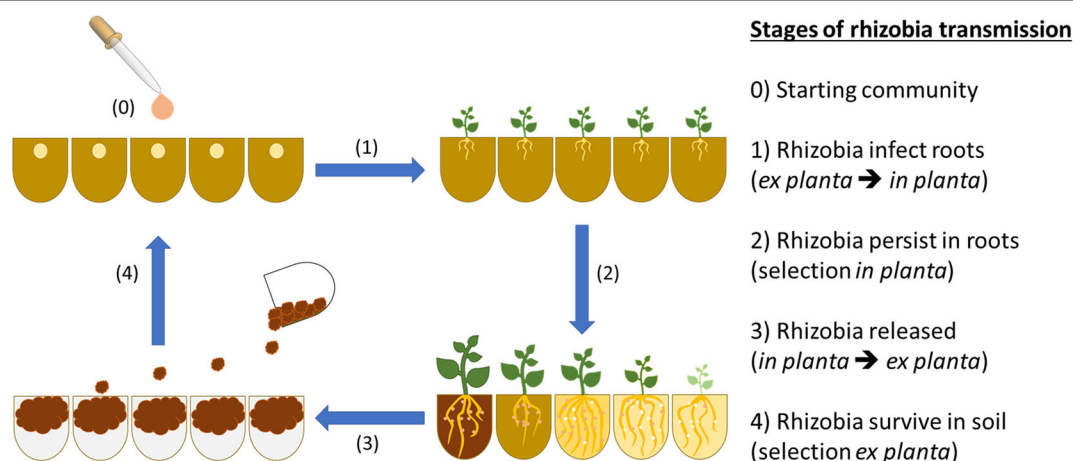
face a secondary issue of selection in the soil, which is vastly different from selection *in planta* (Burghardt et al. 2018; Kaminsky et al. 2019). Resource scarcity in the soil results in selection pressure to use diverse carbon sources (Hollowell et al. 2016), and antibiotic production by diverse soil microbes imposes selection on microbes that are resistant or susceptible to these antibiotics (Hollowell et al. 2015). Moreover, spatio-temporal differences in host availability can amplify interspecific microbial competition through continuous fluctuations in the dominant rhizobia species present (Pahua et al. 2018). Microbiome engineering predicts that legume mechanisms are sufficient to control rhizobia infection and persistence, but selection pressures related to released rhizobia and their survival in the soil are susceptible to passaging methodology during microbiome engineering.

The microbiome engineering methodology we describe is aimed at studying the RCP through changes to rhizobial species abundance and rhizobia allele frequencies. Importantly, this methodology differs from previous microbiome engineering by outlining *the introduction of various rhizobia to an existing soil microbial community that could contain both native and naturalized rhizobia species*. The initial bulk soil can have any physical, chemical, and microbial composition. Sterilized soil used throughout will minimize changes to the physical and chemical properties of the soil, and the initial microbial community can be extracted from pre-sterilized bulk soil (Lau and Lennon 2011; Panke-Buisse et al. 2015). The use of

individual pot replicates maintains microbiome independence for the eventual selection of potted plants based on seedpod production. We suggest selecting for seedpod production because a large number of legumes are grown for seedpods (Rawal and Navarro 2019), and because the production of seedpods corresponds to the release of rhizobia from senescing nodules (Puppo et al. 2004). The soil microbiome can be passed once pots with desired levels of seedpod production have been determined. Rapid passaging is required to mitigate drastic alterations to the soil microbiome (Burghardt et al. 2018; Kaminsky et al. 2019; Zgadzaj et al. 2016). Microbiome passaging in this methodology is defined as the immediate homogenization of soil from the selected pot with sterilized bulk soil. *The goal for this general methodology is to maintain consistency in the ex planta life of the rhizobia (stages 3 and 4), while the following approaches modify the initial inoculum as a way to study in planta rhizobia (stages 1 and 2).*

### Commercially available inoculum approach

The first approach is the most similar to current agricultural practices and a direct test of the classic rhizobial competition problem. In addition to the extracted soil community, the initial inoculum includes a commercial elite rhizobial inoculant. Assuming elite rhizobia can successfully infect a subset of hosts, the superior



**Fig. 2** The four stages of rhizobia transmission. Rhizobia are soil dwelling bacteria that are commonly associated with legumes, plants of great agronomic importance. (1) Rhizobia regularly induce beneficial infections on legume roots that result in the formation of a nodule, a specialized organ that legumes use to control resource flow and *in planta* rhizobial population size. (2) Within these nodules, rhizobia are supplied with photosynthates, which they use to increase population size and provide the necessary energy to fix atmospheric nitrogen. Fixed nitrogen is then

used by host legumes which can substantially increase plant growth. (3) As legumes approach flowering, nodules gradually begin to senesce as resources are redirected to seed production, and rhizobia are released into the soil. (4) Rhizobia then reside within the soil until they infect a new host. Microbiome engineering leverages all stages of transmission. Stage 3, in particular, is when released rhizobia are transferred to new soil. Note the color of the soil transferred between stages 3 and 4 represents the soil from the largest plant

nitrogen fixation of elite rhizobia is predicted to drastically increase their relative abundance *in planta* compared to soil rhizobia through host control mechanisms that select against less beneficial rhizobia (stage 2; Denison 2000; West et al. 2002). Thus, the aim of this approach is to explore mechanisms that increase elite rhizobia infection (stage 1) and infer implications for the RCP.

Natural selection is predicted to favor rhizobia that can infect legume roots, as this can dramatically increase population size compared to life in the soil (Denison and Kiers 2004). Rhizobial infection of legume roots is canonically attributed to their molecular dialogue, but other traits can also facilitate infection. Biofilm formation enhances root colonization, thus increasing infection rates (Rinaudi and Giordano 2010). Additionally, infection and nodulation of soybean has been linked to the presence of the soybean *Rj4* resistance allele (Tang et al. 2016). However, mutant rhizobial symbionts overcome this resistance, which result in high rates of nodulation (Faruque et al. 2015). Similarly, there is evidence that rhizobia of more recently evolved symbioses can bypass host mechanisms that limit infection by reverting to ancient mechanisms of legume root infection, such as crack entry (Acosta-Jurado et al. 2016; Okazaki et al. 2013) or peg infection (Kawaharada et al. 2017).

Moreover, non-nodulating rhizobia have been identified within root tissue that have endophytic behavior (Zgadzaj et al. 2015; Gano-Cohen et al. 2016). While these mechanisms are often associated with parasitic rhizobia, they serve as examples of rhizobia traits that could evolve in elite rhizobia to increase access to host tissue.

Strategies to increase elite rhizobia infection are at the core of solving the RCP. While the ultimate goal is to increase elite rhizobia access to host tissue, we should not ignore host-mediated mechanisms that prevent excessive nodulation to optimize host growth (i.e., autoregulation of nodulation; Quides et al. 2021; Sachs et al. 2018). We can mitigate issues related to excessive nodulation by implementing microbiome engineering that allows us to select microbiomes based on host seedpod production. Selecting soil microbiomes that result in the greatest seedpod production, irrespective of any shifts in microbiome or mutations, could favor communities that maximize relative abundance *in planta* of elite rhizobia. Changes to the soil community are not dependent on identifying mutations in the elite rhizobia, as a single generation experiment suggested elite inoculum have a strong influence on *in planta* rhizobial diversity (Gerding et al. 2014). Shifts in microbiome composition can also promote increased infection by



elite rhizobia. Next, we present an alternative perspective for generating solutions to the RCP.

### Hyper-competitive inoculum approach

Prior strategies to mitigate the RCP have largely focused on promoting infection by rhizobia in elite inoculants (Santos et al. 2019), but direct competition for shared host resources can extend competition beyond infection (stage 1) and into persistence (stage 2). Host control mechanisms are predicted to be sufficient to select against sub-optimal rhizobia, but in some cases, rhizobia that extract a disproportionate amount of resources can persist within host tissue (Gano-Cohen et al. 2016; Kiers et al. 2006; Quides et al. 2017; Sachs et al. 2010; Zgadzaj et al. 2015). By limiting the energetic output for nitrogen fixation, rhizobia can increase host resource consumption for their own reproduction (Trainer and Charles 2006). The resulting composition of *in planta* mixed-infection rhizobia could then appear as though elite rhizobia had limited infection success, when in fact, the problem lays with persistence, and therefore abundance, compared to soil rhizobia. Persistence *in planta* by rhizobia that provide little to no benefit is likely to contribute to the RCP, but traits that promote persistence are still unclear. Here, we suggest the use of hyper-competitive rhizobia to maintain consistent infection (stage 1) in order to focus on mechanisms that allow for persistence *in planta* (stage 2). We outline two different approaches that utilize hyper-competitive rhizobia to study prevention or disruption of host control mechanisms.

One strategy for rhizobia persistence *in planta* is to prevent the activation of host control mechanisms altogether. To explore this set of strategies, a hypercompetitive rhizobium that has demonstrated moderate to minimal levels of nitrogen fixation or host growth benefit is best suited. By starting with a moderately beneficial genotype, negative or positive selection for benefit provided by rhizobia could cross a threshold to trigger host control mechanisms. Many legumes have mechanisms that select against *in planta* rhizobia based on the benefit they provide (Sachs et al. 2018), but the rhizobia-derived stimulus that triggers host control mechanisms remains unresolved. There is growing evidence that rhizobia polysaccharides are a key component in communication beyond initial infection (Via et al. 2015). For example, rhizobia lipopolysaccharides play a key

role in inhibiting the soybean immune response against rhizobia symbionts (Margaret et al. 2013). Additionally, amino acid exchange might serve as a metabolic proxy for benefit, leaving host control mechanisms susceptible to misinterpretation of the benefit provided by rhizobia (Lodwig et al. 2003; Molero et al. 2014; Prell et al. 2009). More generally, soil rhizobia that provide moderate levels of benefits have been recovered from nodules at population sizes consistent with highly beneficial rhizobia (Sachs et al. 2010), suggesting host control mechanisms were not triggered by the host used in this experiment. By starting with hypercompetitive rhizobia that can provide benefit, this approach can identify rhizobia traits associated with the stimulus for host control mechanisms, which in turn, may explain how rhizobia persist *in planta*.

Disruption of host control mechanisms has been proposed as an additional strategy for rhizobia to increase persistence within host tissue. In order to promote host control mechanisms, and subsequent rhizobia disruption traits, a hyper-competitive rhizobium that cannot fix nitrogen should be used. One of the best described rhizobia disruption mechanisms occurs in hosts that produce NCR peptides. These host produced peptides limit proliferation of rhizobia *in planta*, but rhizobia-derived enzymes have been discovered that destroy these peptides and allow rhizobia to reproduce (Alunni and Gourion 2016). Another proposed host control mechanism is the premature senescence of nodules through programmed cell death (Cam et al. 2012; Regus et al. 2017). This mechanism differs from senescence associated with changes in phenology by being highly localized, which does not compromise the structure of the nodule. Rhizobia have been shown to scavenge resources after natural nodule senescence (Timmers et al. 2000), which represents a potential persistence strategy within intact nodules that have partially senesced. Additionally, flavodoxin, an electron transfer protein, has been shown to delay the senescence of nodules (Redondo et al. 2009). Flavodoxin was cloned into the rhizobia from the nitrogen fixing *Anabaena variabilis* cyanobacterium, thus it remains possible that an analogous rhizobia protein exists. Part of the difficulty with identifying these rhizobial traits stems from our limited understanding about the molecular mechanisms that drive host control, but this should not limit our ability to identify rhizobia traits that contribute to rhizobia persistence.

Hyper-competitive rhizobia are readily isolated from the soil (Irisarri et al. 2019), however, the level of benefit they provide can vary greatly (Bourion et al. 2018; Koskey et al. 2017; Pastor-Bueis et al. 2019; Soe et al. 2020). Hyper-competitive rhizobia that provide high levels of benefit represent one approach to overcome the RCP, and these strains are commonly proposed for future elite inoculants (Kaminsky et al. 2019). Here, the focus on hyper-competitive rhizobia that provide little to no benefit can help us understand the barriers preventing us from solving the RCP. Both of these microbiome engineering approaches using hyper-competitive rhizobia provide the option to passage the soil microbiome of pots that produce the most or least seedpods. Passaging the most productive pot, when starting with a low benefit rhizobia, could identify microbiomes that have evolved to provide increased benefit, either through increased provisions (Remigi et al. 2016), or extended persistence to the point of providing benefit (Schumpp et al. 2009). Alternatively, passaging of the least productive pot in either approach could identify additional exploitative phenotypes related to the activation of host control mechanisms or disruption of host control. In this case, microbiome engineering can offer guidance when exploring the role of soil rhizobia in the RCP. Although the specifics of host control mechanisms are unknown, changes to the microbiome may hint at potential mechanisms.

### Synthetic community inoculum approach

The final approach integrates synthetic microbial communities with microbiome engineering. Synthetic communities are predicted to have a greater chance of an elite rhizobia infecting compared to a clonal inoculant, and they can be designed to include beneficial microbes in addition to nitrogen fixing rhizobia (Santos et al. 2019). We discuss two options for synthetic communities to study the RCP, an introduced community of only rhizobia and a community that includes rhizobia and other microbe species. These synthetic communities differ from the previous two approaches, in that they are not aimed at studying a specific stage. Rather, the use of a synthetic community will offer insight into the cumulative effects of the legume-rhizobia interaction on rhizobia displacement, survival, and integration with the soil microbial community.

Introducing multiple species of rhizobia to the soil can increase the likelihood that one elite rhizobia species can survive, infect, and provide benefit to the target legume. The most common practice is the use of two rhizobia species (Santos et al. 2019), but increasing this to dozens of species provides greater genetic variation to explore broad patterns of how rhizobia interact with legumes and their environment. For instance, a synthetic community of 101 strains of rhizobia experienced large variation in selection pressure when grown *in vitro*, in soil, and in the presence of two different host legumes (Burghardt et al. 2018). In this study, the synthetic community of rhizobia were only exposed to hosts for one generation and did not compete with soil rhizobia, but the authors provide an elegant framework to use synthetic communities for microbiome engineering. Starting with a large, traceable community allows for the identification of common traits that increase infection rates, persistence *in planta*, release from host tissue, and survivorship in the soil between infections. This could also be implemented to help us understand how introduced rhizobia interact with established soil microbiomes. Native and naturalized microbes are well adapted to their environment, but that does not make them immune to invasion by an influx of new cohabitants.

Rhizobia are commonly referred to as the sole providers of benefit to legumes, but the soil microbiome is extremely diverse and contributes a multitude of benefits (Friesen et al. 2011). Recent developments in inoculant design consider this by incorporating microbes that produce phytohormones, increase access to phosphate, or prime host immune systems against pathogens (Santos et al. 2019). For example, inoculating arbuscular mycorrhizal fungi with rhizobia have been shown to increase nodulation and shoot growth (Larimer et al. 2014; Ossler et al. 2015; Omirou et al. 2016), but it is unclear if this also increases host susceptibility to the RCP by increasing rhizobia access to host tissue. Additionally, legume host control mechanisms are thought to share similar pathways with the innate immune response (Tóth and Stacey 2015), thus microbes that prime the immune system may play a complimentary role by suppressing infection and persistence by minimally beneficial rhizobia that are involved in the RCP. Identifying the core microbiome for agriculture crops still remains a long-term goal (Busby et al. 2017), but experiments that use elite rhizobia alongside microbes that modulate host growth and immunity will



help us elucidate synergistic qualities that suppress infection and persistence of soil rhizobia that provide minimal benefit.

The use of a synthetic community to study the RCP provides us with a macroscopic view of the legume-rhizobia interaction. The benefit of a synthetic community compared to a natural community is that we can design a known community with varying degrees of complexity and then track the community over time. The starting microbial community will likely dictate the goals of a study and these goals should be used to determine which soil microbiome to passage. For example, a community composed of nitrogen fixing rhizobia, disease suppressing *Trichoderma* sp., and phosphate acquiring mycorrhizae may wish to passage the most productive pots to examine how integration of this synthetic community leads to increased legume growth. Alternatively, passaging the least productive pots from a synthetic community of rhizobia that fix little to no nitrogen could help us find traits associated with displacement of rhizobia. Creating synthetic soil microbiomes is still gaining momentum (Busby et al. 2017), but with advances in next generation sequencing and an abundance of existing sequenced soil microbes, we have the tools to create increasingly complex synthetic microbial communities that can be tracked through multiple generations.

### The complementary role of sequencing

High-throughput sequencing has become increasingly accessible and plays a key role in adequately tracking changes while engineering microbiomes. The plethora of multi-omics approaches has recently been reviewed for the legume-rhizobia symbiosis (Dicenzo et al. 2019). Here, we describe recent sequencing approaches and we discuss considerations to take when sampling microbiomes while implementing microbiome engineering to study the RCP.

Next generation sequencing is imperative to rapidly observe ecological and evolutionary dynamics in soil microbiomes. Sequencing *nodD* has been used to track diverse symbiotic rhizobia (Boivin et al. 2020), 16S rRNA sequencing can detect bacterial changes to the microbiome (Zgadzaj et al. 2016), and fungi can be tracked with internal transcribed spacers sequencing (Thiergart et al. 2019). Additionally, rhizobial strains can be distinguished with chromosomal genes and symbiosis genes (Hollowell et al. 2016). This is useful if the

goal is to identify broad shifts in a microbiome with different starting inoculum, but it would be nearly impossible to track individual species through the microbiome engineering process. Burghardt and colleagues (2018) demonstrated a presequence, select, and resequence framework that can track strains within a population and identify single nucleotide polymorphisms, but incorporating additional approaches are required to track species through complex soil communities composed of diverse genera (Albanese and Donati 2017). Similarly, innovative uses of plasmid-tagged rhizobia in conjunction with green fluorescent protein have allowed *in vivo* detection of competitiveness and nitrogen fixation of multiple rhizobial species simultaneously (Mendoza-Suárez et al. 2020). These methodologies can largely capture ecological shifts in the microbiome, but the influence of mobile genetic elements in the soil microbiome should also be considered.

Horizontal Gene Transfer (HGT) can provide bacteria with symbiotic genes, but they will often lack the complete suite of symbiosis genes. The resultant novel symbiont will therefore lack mutualistic genes, while providing access to host tissue and resources. HGT is a natural process (Bamba et al. 2019), but can be problematic in legume crop fields. Brazil has experienced issues with native rhizobia acquiring inoculant symbiotic genes contributing to the RCP for Soybeans (Barcellos et al. 2007) and the same phenomenon has been reported for Soybeans grown in Europe (Yuan et al. 2020). Conversely, reports of HGT in Australia have not impacted chickpea production (Elias and Herridge 2015). The role of HGT in the RCP is likely substantial, and the relative ease with which we can sequence symbionts will allow us to better predict symbiotic qualities of novel symbionts generated through HGT. With advances in next generation sequencing, we now have *accessible tools to track broad changes to the microbiome and specific changes to microbial genes of interest* that were previously cumbersome and costly.

To effectively monitor the legume microbiome through the engineering process, it is important to recognize the continual cycle of *in planta* and *ex planta* life for rhizobia. Tracking the microbiome is best suited for *ex planta* sampling because sequencing often involves disruptive, if not destructive, sampling. Moreover, the rhizobia that we are interested in studying for the RCP must complete the *ex planta-in planta-ex planta* cycle. The RCP is defined by sub-optimal rhizobia present *in planta*, but these sub-optimal rhizobia also must escape

from one host to infect a new host. We suggest sampling during stage 3 and stage 4 of rhizobia transmissions for each passage to track ecological and evolutionary dynamics of the microbiome. Stage 3 in our passing protocol is before homogenization with soil, and stage 4 is at the time of sowing seeds (Fig. 2). By sampling at both stages, we can capture and account for changes due to do homogenization. If there are core microbial species, traits, or genes that enable infection and persistence, we would expect to see them appear in stage 4 of one passage, and stage 3 of the next.

### Concluding thoughts and future directions

We outlined a series of microbiome engineering approaches that we hope will spur new research on the rhizobial competition problem that has long impeded our ability to move beyond expensive and polluting chemical fertilizers (Table 1). Despite microbiome engineering gaining popularity, we know of only one study that has attempted to engineer legume microbiomes (Anand et al. 2020). A series of rhizobia experimental evolution studies (Guan et al. 2013; Marchetti et al. 2010, 2014), which share many of the same properties as microbiome engineering, offer a clue to the difficulties that may be encountered when engineering legume microbiomes. Although these studies demonstrated a pathogen evolved to nodulate a legume after HGT, nitrogen fixation was not attained (Guan et al. 2013; Marchetti et al. 2010, 2014). Moreover, the evolutionary processes observed through experimental evolution were found to be similar to natural processes (Clerissi et al. 2018). Microbiome engineering studies may encounter a similar problem, whereby clonal legumes struggle to maintain efficient host control mechanisms against rapidly evolving symbionts (Remigi et al. 2014, 2016). This does not diminish the impact microbiome engineering can have when studying the RCP, but *we should not ignore the effect of plant hosts on plant-microbe interactions* (Porter and Sachs 2020). As we continue to explore the evolutionary history of domesticated crops (Porter and Sachs 2020), we can study the real-time evolution of soil microbiomes in parallel.

Application of microbiome engineering at an agricultural scale would be extremely difficult, but simplified microbiome engineering can be used on a site-by-site basis to identify context dependent contributors to the

**Table 1** Overview of microbiome engineering approaches described

Initial inoculum	Rhizobia to be studied	Rhizobia stage of transmission to be studied	What should be passaged?	Hypothesis related to the Rhizobial Competition Problem
Bulk soil microbes	All	Stage 1–4	Most productive	Baseline test of host control mechanisms effect on the microbiome
Commercial + bulk soil microbes	Commercial	Stage 1	Most productive	Commercial rhizobia struggle to infect nodules and are recovered from nodules at low rates
Hyper Fix+ + bulk soil microbes	Hyper Fix+	Stage 2	Most OR least productive	Hyper-competitive rhizobia persist within nodules by preventing the activation of host control mechanisms and are recovered from nodules at high rates
Hyper Fix- + bulk soil microbes	Hyper Fix-	Stage 2	Least productive	Hyper-competitive rhizobia persist within nodules by disrupting host control mechanisms and are recovered from nodules at high rates
Synthetic community + bulk soil microbes	All	Stage 1–2	Most OR least productive	Rhizobia species and microbial species have variable effects on host control mechanisms that alter the legume-rhizobia interaction

RCP (Vessey 2004). For example, a rhizobia strain was isolated from a field in Germany that provides greater benefit to Soybean than an elite inoculant at near freezing temperatures (Yuan et al. 2020). This suggests that beneficial rhizobia were already present in this soil, and microbiome engineering could be used to determine if, and how, the soil community could be shaped by Soybeans to enhance growth and seed pod production in cold conditions. A complementary approach could work with soil that has a history of the RCP (Alves et al. 2003; Barcellos et al. 2007; Ferreira and Hungria 2002) by engineering a microbiome under variable conditions that facilitates *in planta* and *ex planta* persistence of elite inocula while maximizing Soybean productivity. Alternatively, regions that have been less impacted by the RCP (Elias and Herridge 2015) may wish to engineer microbiomes to anticipate the effect of future climatic changes on elite inocula productivity. For example, a microbiome engineering approach was used with *Vigna radiata* to successfully coalesce a soil community that provides saline tolerance (Anand et al. 2020). This demonstrates the utility of microbiome engineering and future work can identify specific shifts in microbial abundance or allelic change to the soil microbiome that confer the observed benefit.

Recent developments in molecular tools (Dicenzo et al. 2019), and creative methodologies (Mueller and Sachs 2015) have made finding a solution to the RCP more attainable. Single generation experiments have demonstrated how we can track microbiomes through multiple generations (Gerding et al. 2014; Zgadzaj et al. 2016; Burghardt et al. 2018; Mendoza-Suárez et al. 2020), and future studies have the potential to continue progressing towards solving the RCP. In addition to the general framework described here, we encourage future work to vary the approaches we describe to best address the goals of a study. For instance, if studies are focused on inoculum evolution, soil microbes could be excluded. Inert growth media, such as sand, calcined clay, or hydroponics, could be used if the aim is to look at the effect of specific chemicals. To explore *ex planta* selection, soil microbiomes could be left in stage 4 for an extended period. Lastly, this microbiome engineering approach could be adapted to experimental evolution by removing the artificial selection phase and running multiple independent experimental lineages. Microbiome engineering is not a one-size-fits-all methodology to answer all our questions about the legume-rhizobia symbiosis, but it provides us with a highly flexible framework. While we

have a sturdy foundation of knowledge for both legumes and rhizobia, there is still much we need to learn about how these partners interact.

The methods we describe here are not meant to be all inclusive. Rather, we highlight the diverse utility of microbiome engineering to create a scaffold for future work. The ecological phenomena of the rhizobial competition problem involves a complex array of host and symbiont mechanisms, which cannot be overcome by simple solutions. Future work will need to innovate on existing approaches as we continue to learn about the complexities of the legume microbiome. We now can observe microbial evolution as it unfolds, an opportunity we should not take for granted as we continue to learn about the seemingly endless role of microbiomes in everyday life.

**Abbreviations** RCP, Rhizobial Competition Problem; HGT, Horizontal Gene Transfer

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