Title: A simple assay for quantification of plant-associative bacterial nitrogen fixation

Running title: Quantification of associative nitrogen fixation

Authors: Timothy L Haskett[1*] & Philip S Poole[1]

Affiliations: [1]Department of Plant Sciences, University of Oxford, Oxford, OX1 3RB, UK1

[*]Author for correspondence timothy.haskett@plants.ox.ac.uk

Abstract

Accurate quantification of plant-associative bacterial nitrogen (N) fixation is crucial for selection and development of elite diazotrophic inoculants that could be used to supply cereal crops with nitrogen in a sustainable manner. Because a low oxygen environment that may not be conducive to plant growth is essential for optimal stability and function of the N-fixing catalyst nitrogenase, quantification of N fixation is routinely carried out on "free-living" bacteria grown in the absence of a host plant. Such experiments may not divulge the true extent of N fixation occurring in the rhizosphere where the availability and forms of nutrients such as carbon and N, which are key regulators of N fixation, may vary widely. Here, we present a modified *in planta* acetylene reduction assay, utilising the model cereal barley as a host, to quantify associative N fixation by diazotrophic bacteria. The assay is rapid, highly reproducible, applicable to a broad range of diazotrophs, and can be performed with simple equipment commonly found in most laboratories that investigate plant-microbe interactions.

Importance

Exploiting “nitrogen-fixing” bacteria that reduce atmospheric dinitrogen into ammonia as inoculants of cereal crops has great potential to alleviate current inputs of environmentally deleterious fertiliser nitrogen and drive more sustainable crop production. Accurately quantifying plant-associative bacterial nitrogen fixation is central to the development of such inoculant bacteria, but most assays fail to adequately reproduce the conditions of plant root systems. In this work, we have validated and optimised a simple *in planta* assay to accurately quantify N fixation in bacteria occupying the root and surrounding soil of the model cereal barley. This assay represents a benchmark for quantification of plant-associative bacterial N fixation.

Introduction

Exploiting diazotrophic bacteria that reduce atmospheric dinitrogen (N_2) into ammonia (NH_3^+) as inoculants of cereal crops has great potential to alleviate current inputs of environmentally deleterious fertiliser nitrogen (N) in agricultural systems to establish more sustainable crop production (1). While a plethora of diazotrophic strains have been isolated that colonise the root compartments (rhizosphere, rhizoplane, and endosphere) of cereals (2), it remains unclear which strains are best suited to agriculture. Elite inoculants should ideally a) competitively colonise and persist in plant root compartments to exert their beneficial effects, b) exhibit some degree of interactive specificity with the target host to prevent promiscuous growth promotion of non-target species, and c) fix and release large quantities of N for assimilation by the plant (3). While no natural bacteria have been categorically demonstrated to satisfy these three criteria, targeted selection and genetic engineering programs are currently underway to assist in the development of elite inoculant strains (2, 4-8).

Quantification of bacterial N fixation is central to the selection and development of elite inoculant strains and is typically carried out using either an acetylene reduction assay (ARA) or ^{15}N incorporation assay. ARAs rely on the use of GC-MS to monitor the alternative ability of the N-fixing catalyst nitrogenase to reduce acetylene (C_2H_2) to ethylene (C_2H_4) (9), whereas ^{15}N incorporation assays can be used to track assimilation of fixed ^{15}N by bacteria or even by plants following bacterial release of NH_3^+ (10-12). Because a low oxygen (< 10%) environment that may not be conducive to plant growth is essential for optimal nitrogenase stability and function (13), both assays are best suited to analysis of bacterial cultures grown in the absence of a host plant. However, this condition may not reflect the true extent of N fixation occurring in the rhizosphere where the availability and forms of nutrients such as carbon (C) and N, which are key regulators of N fixation (5, 14-16), may vary widely. A benchmark assay is needed to assess associative bacterial N fixation in a manner that more accurately reflects conditions in the rhizosphere.

Here, we present a simple *in planta* ARA to quantify N fixation in diazotrophic bacteria occupying the root system of the model cereal barley (Fig 1). We demonstrate that the assay is highly

reproducible, rapid, applicable to genetically diverse diazotrophs, and requires minimal equipment commonly found in laboratories investigating plant-microbe interactions.

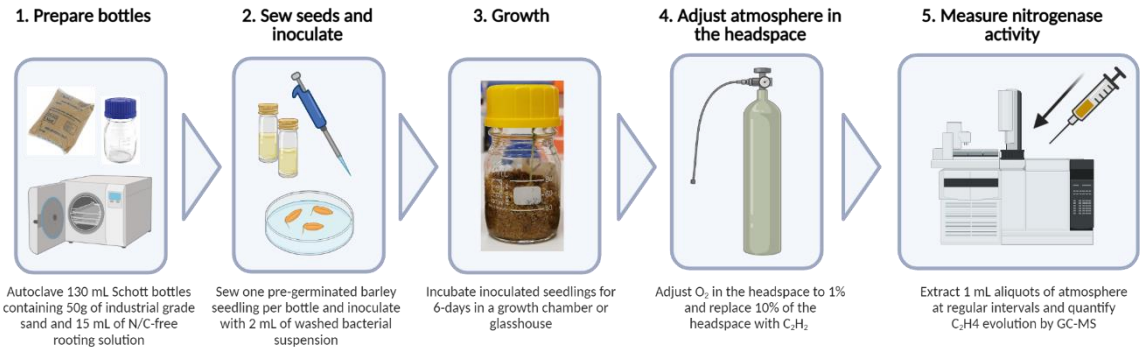

Figure 1. Schematic of workflow for *in planta* acetylene reduction assay. This figure was created with BioRender®.

Results

Validation of *in planta* ARA. To both validate and optimise our *in planta* ARA described (Fig 1), we utilised the cereal endophyte and *Sesbania rostrata* nodulating symbiont *Azorhizobium caulinodans* ORS571 (*Ac*) as a model strain. The assay was initially set up by sewing individual pre-germinated, surface-sterilised Barley seeds into 130 mL Schott bottles containing industrial grade sand and N/C-free rooting solution, inoculating the plants with 2 mL of an OD_{600nm} 0.1 suspension of *Ac* (approximately $5x10^7$ cells), then growing the plants in a growth-chamber for 6-days. At this point, the atmosphere in the headspace was adjusted to 1% O_2 and Schott bottles were sealed with a rubber septum. Ten percent of the headspace was next replaced with C_2H_2 and plants were returned to the growth chamber. The reduction of C_2H_2 to C_4H_4 by nitrogenase was measured over 72-h using GC-MS. In all five biological replicates, significant C_2H_4 production (69.97 $\pm$ s.e.m 17.05 nmoles C_2H_4 h^{-1} $plant^{-1}$) was detected after 24-h and the subsequent rates of C_2H_4 production remained stable up to 72-h with a mean rate of nitrogenase activity of 55.57 $\pm$ s.e.m. 11.23 nmol C_2H_4 h^{-1} $plant^{-1}$ between 24 and 48-h, and 62.77 $\pm$ s.e.m. 14.11 nmol C_2H_4 h^{-1} $plant^{-1}$ between 48 and 72-h (Fig 2A). Using the above conditions, we also confirmed that neither the plant nor bacteria exhibited nitrogenase activity

in the absence of the symbiotic partner between 0 and 72-h (Fig 2B). Thus, bacterial N fixation monitored in the assay was entirely dependent on nutrients provided by the host plant.

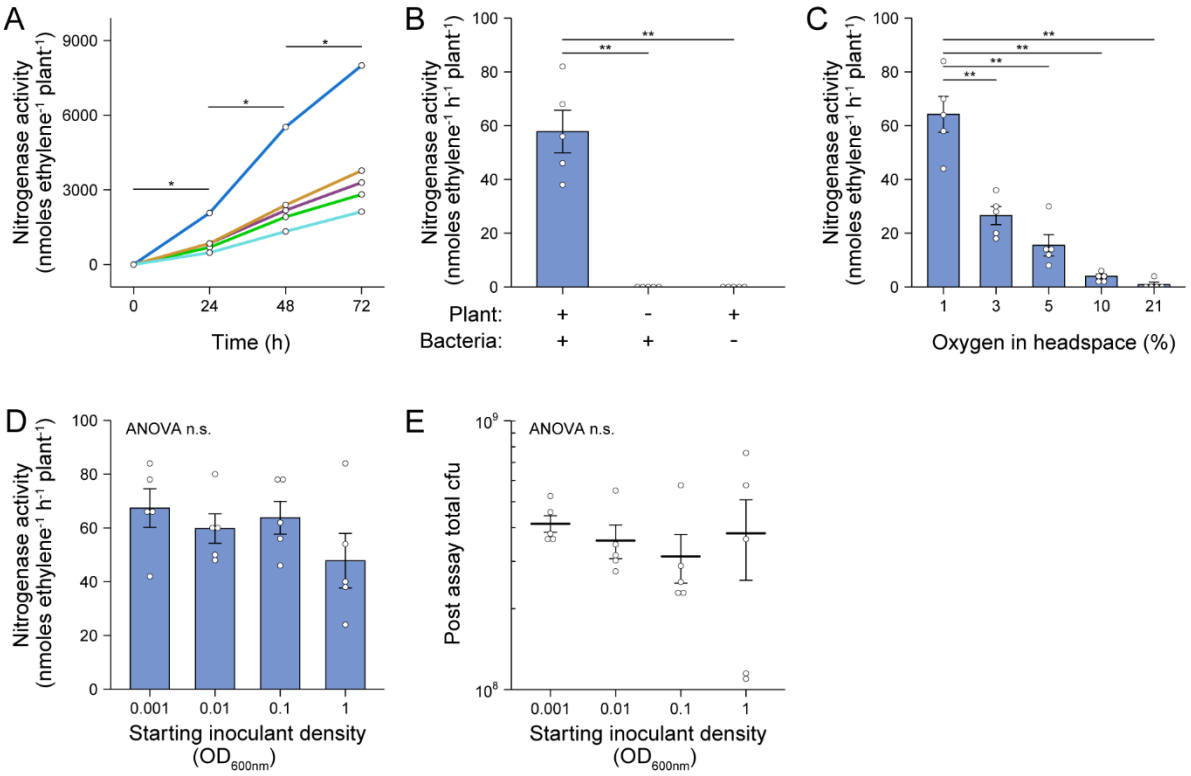

Figure 2. Validation and optimisation of *in planta* acetylene reduction assay. *A. caulinodans* ORS571 (*Ac*) was used as the model strain for these experiments. The assays were set up by sewing individual pre-germinated, surface-sterilised barley seeds into 130 mL Schott bottles containing industrial grade sand and N/C-free rooting solution, inoculating the plants with 2 mL of an OD_{600nm} 0.1 suspension of *Ac*, then growing the plants in a growth-chamber for 6-days. At this point, the atmosphere in the headspace was adjusted to 1% O_2, the bottles were sealed with a rubber septum and 10% of the headspace was replaced with C_2H_2. Bottles were returned to the growth chamber and the reduction of acetylene (C_2H_2) to ethylene (C_4H_4) by nitrogenase was measured at 24-intervals over 72-h using GC-MS. Mean ± s.e.m (error bars) and individual values for five biological replicates are plotted. Nitrogenase activity was calculated between 24-h and 48-h. ANOVA and pairwise two-tailed students t-tests with Bonferroni adjusted P-values were used to compare means where relevant. $P > 0.05$ not significant (n.s.), $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***. **(A)** C_2H_4 production in each of five biological replicates was monitored over 72-h. **(B)** Rates of nitrogenase activity where measured when the plant or bacteria was omitted from the system. **(C)** Rates of nitrogenase activity were measured when the starting O_2 concentration in the headspace was adjusted between 1% and 21% (i.e. air) by flushing with N_2 gas. **(D)** Rates of nitrogenase activity were measured when the starting inoculant density was adjusted between OD_{600nm} 0.001 to 1. **(E)** Total colony forming units (cfu) present in the assay systems of experiment (D) as determined by viable counts after 72-h.

Optimisation of *in planta* ARA. We further optimised our *in planta* ARA first by titrating the starting O_2 concentration in the headspace of Schott bottles after 6-dpi. We found that an optimum

rate of nitrogenase activity, similar to that of the experiments in Fig 2A-B, was observed where O_2 in the headspace was adjusted to 1% of the atmosphere (Fig 2C). However, low nitrogenase activity was also observed where the O_2 concentration was adjusted to 10% (3.98 $\pm$ s.e.m. 0.83 nmol C_2H_4 h^{-1} $plant^{-1}$) and even 21% (0.89 $\pm$ s.e.m. 1.98 nmol C_2H_4 h^{-1} $plant^{-1}$), although the latter rate was the product of a single biological replicate out of five (Fig 2C).

We next tested the effect of titrating the starting inoculant density between OD_{600nm} 0.001 and 1.0 and found that nitrogenase activity did not differ between these treatments (Fig 2D). Moreover, when bacteria were recovered at the close of the assay by rigorous flushing with PBS, the total number of colony-forming units (cfu) in each Schott bottle did not differ significantly, with each harbouring between 10^8 and 10^9 cfu (Fig 2E). This suggested that after 6-dpi, *Ac* naturally reaches the carrying capacity of the assay system.

Test of *in planta* ARA on genetically diverse diazotrophic bacteria. To explore whether our *in planta* ARA could be used to quantify associative N fixation by diazotrophs other than *Ac*, we selected the following seven additional alpha-, beta- and gamma-proteobacterial strains for testing; *Azospirillum brasilense* FP2 (*Ab*), *Azoarcus olearius* DQS-4 (*Ao*), *Burkholderia vietnamensis* WPB (*Bv*), *Herbaspirillum seropedicae* SmR1 (*Hs*), *Klebsiella oxytoca* M5a1 (*Ko*), *Pseudomonas stuzeri* A1501 (*Ps*) and *Rhodobacter sphaeroides* WS8 (*Rs*). Plants were inoculated with 2 mL of an OD_{600nm} 0.01 suspension and after 6-dpi, the atmosphere in the headspace was adjusted to 1% O_2 and 10% C_2H_2 to begin the assay. GC-MS measurements for C_2H_4 production were made at 12-h intervals over 72-h. For most of the strains, nitrogenase activity was detectable by 24-h, but a stable, optimal rate of nitrogenase activity required at least 36-h of preparation (Fig 3A). Mean rates of nitrogenase activity were measured for all strains between 48 – 62-h (Fig 3B), with the highest rates between 58 and 65 nmol C_2H_4 h^{-1} $plant^{-1}$ for *Ao, Ps, Ac,* and *Ab.* The remaining strains fixed between 7 and 30 nmol C_2H_4 h^{-1} $plant^{-1}$, whereas no nitrogenase activity was detected in the uninoculated controls. The range in nitrogenase activity displayed by the eight diazotrophic bacteria tested highlights that some bacteria are better adapted to associative N fixation with barley then are others.

We also recovered bacteria from the assays and performed viable counts to estimate the total number of cfu. Interestingly, *Ao*, which exhibited the equal highest nitrogenase activity, was found to be the least abundant in the barley root systems (Fig 3C), indicating that individual cells may be capable of fixing a considerable amount of N relative to the other strains. However, we suspect that this result may be influenced by poor viability of the bacterium upon recovery from the plant, as has previously been documented (17). Conversely, the strains which were most abundant in the barley root system, *Bv* and *Ko*, exhibited the equal poorest nitrogenase activity, suggesting that these strains are well-adapted for proliferation in the barley rhizosphere, but poorly adapted to N fixation in this environment.

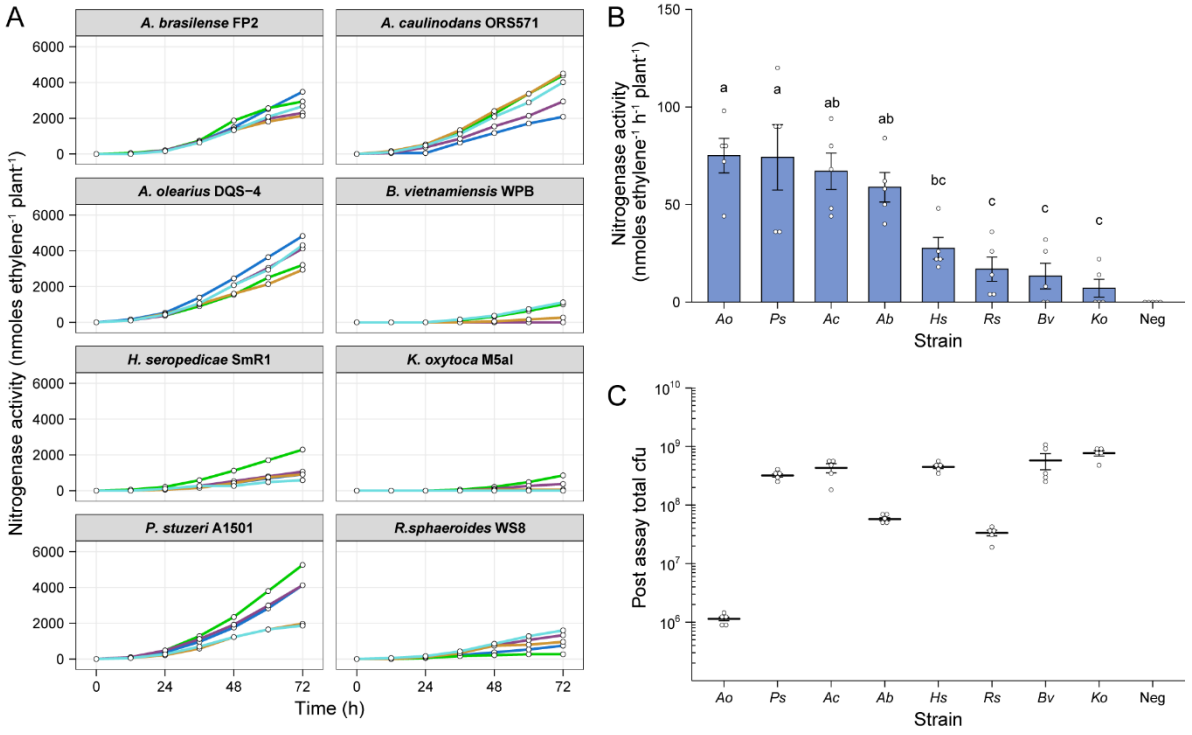

Figure 3. Comparison of associative nitrogen fixation between eight model bacteria. Assays were set up as described in Fig 2. Two millilitres of an OD_{600nm} 0.01 suspension of bacteria were used for inoculation and C_2H_4 production was measured at 12-h intervals over 72-h. **(A)** C_2H_4 production was measured for five biological replicates of eight genetically diverse diazotrophic bacteria inoculated gnotobiotically onto barley. **(B)** The mean ± s.e.m (error bars) rates of nitrogenase activity between 48-h and 62-h were calculated for the eight model diazotrophs. ANOVA and LSD tests with Bonferroni adjusted P-values were used to compare means. The negative uninoculated control omitted from these tests. Matching letters depict treatments that are not significantly different ($P > 0.05$) from each other but are significantly different ($P < 0.05$) from treatments with a distinct letter. **(C)** Mean ± s.e.m (error bars) of total cfu present in the assay systems as determined by viable counts after 72-h. Note that the low total cfu count for *Azoarcus olerarius* DQS-4 may be a result of poor viability of the strain upon re-isolation from they plant, as has been reported previously (17).

Discussion

We have demonstrated that our simple *in planta* ARA is highly reproducible, rapid (can be completed in under two-weeks) and is applicable to a diverse range of diazotrophic bacteria. Thus, this assay represents a benchmark for quantification of associative bacterial N fixation.

The simple standardisation involved in our assay workflow (i.e. nitrogenase activity per plant) is one of its most beneficial features, offering a reduced workload compared to other potential approaches. We observed that the total population size of *Ac* in the assay system reached equilibrium after 9-dpi regardless of the starting inoculation density (Fig 2E), and that N fixation was consistent between these treatments (Fig 2C). Nevertheless, there was some variation observed in the total population size when comparing eight genetically diverse endophytes for N fixation (Fig 3). Therefore, in some instances it could be useful to standardise measurements of N fixation on per cell basis. We propose that fluorescent labelling of bacteria in combination with confocal microscopy or flow-cytometry (18) would be a suitable strategy if rates need to be expressed per cell (Fig 2E & Fig 3C) because some bacteria, such as *Ao*, exhibit poor viability upon recovery from plants (17) that could obscure measurements. Dual fluorescent reporter gene fusions could also be made to promoters of the nitrogenase structural gene *nifH* to assess the spatiotemporal dynamics of associative N fixation (19-22). This strategy has been useful in the past to detect *nifH* expression by *A.olerius* DQS-4 on the rhizoplane, endosphere and surrounding soil rice roots when carbon was supplemented into the soil (19, 20).

One of the major constraints of measuring associative N fixation is that optimal nitrogenase activity requires hypoxic conditions of $\leq$ 1% O_2 (Fig 2C) which are detrimental for photosynthesis and plant growth (23). Although long-term exposure of plants to low oxygen ultimately results in anoxia, leading to acidosis and apoptosis, plants can postpone or even prevent tissue from becoming anoxic by tuning the expression or activity of energetically demanding metabolic pathways (24-26) and by producing non-symbiotic leghaemoglobins that help maintain redox status and remove reactive oxygen and nitrogen species (27). Remarkably, in our assay system, N fixation was stable over a 72-h period for all headspace O_2 concentrations tested (Fig 2C), indicating that the plant is still able to

provide adequate nutrients to fuel bacterial N fixation under these conditions. In addition to carbon, bacteria have been engineered to express N fixation genes in response to various plant-derived chemical signals, theoretically imparting upon them a degree of host-specificity for associative N-fixation (3, 8). Assessing such bacteria for associative N fixation using our assay system will be pivotal in the development of these strains, but it remains unclear as to whether plants can sustain production of chemical signalling molecules under low-oxygen stress.

In this work, we utilised barley as a host plant due to the highly uniform growth characteristics of seedings, but also due to its status as a model cereal for engineering the capacity for N fixation (https://www.ensa.ac.uk/) and the availability of a sequenced genome (28). We suspect that the assay could be readily extended to compare N fixation in other host plants, however this may require additional standardisation to account for differences in plant root mass. On the same note, the assay could be readily extended to assess the influence of various abiotic factors, such as plant growth substrates, nutrient levels, pollutants, temperature or light, or be used to explore the influences of abiotic factors on associative N fixation. This could be achieved for example by performing co-inoculation assays or assessing N fixation in non-sterile field soils, although this might be partially impeded by the presence of native N-fixing bacteria. Alternatively, defined synthetic communities of bacteria (29) could be inoculated as competitors for the diazotroph of interest. The validation and optimisation of our assay presented here has paved the way for such future extensions.

Materials and Methods

Preparation of inoculant strains for *in planta* ARA. Bacterial strains used in this study are listed in Table 1. *Klesbsiella oxytoca* was cultured on LB agar (30), *Burkholderia vietnamiensis* was cultured on TY agar (31) and the remaining strains were cultured on UMS agar (32) with 300 μM nicotinate, 10 mM NH_4Cl_2 as a sole nitrogen source, and either 30 mM malate (for *Azoarcus olearius*, *Azospirillum brasilense* and *Herbaspirillum seropedicae*) or 20 mM succinate (for *Azorhizobium caulinodans*) as a sole carbon source. All strains were grown at 28°C, except for *A. caulinodans*

204 which was cultured at 37°C. For *in planta* ARAs, inoculants were prepared by streaking single
205 colonies of bacteria onto agar slopes in 30 mL universal tubes. After 1-2 days incubation, cultures
206 were washed from the slopes three times with PBS to remove residual N and resuspended in N/C-free
207 UMS media at OD_{600nm} 0.001 – 1. The exact $OD600_{nm}$ values are given in the results section for each
208 experiment.

209

210 **Table 1. Bacterial strains used in this study**

Strain	Description	Origin
Azoarcus olearius DQS-4	Betaproteobacterium, isolated from oil-contaminated soil in Taiwan.	(33)
Azorhizobium caulinodans ORS571	Alphaproteobacterium, isolated from *Sesbania rostrata* stems.	(34)
Azospirillum brasilense FP2	Alphaproteobacterium, spontaneous St^R mutant of Sp7 which was isolated from Tropical grasses in Brazil.	(35)
Burkholderia vietnamiensis WPB	Betaproteobacterium, isolated from *Populus* (cottonwood).	(36)
Herbaspirillum seropedicae SmR1	Betaproteobacterium, spontaneous St^R mutant of Z78 which was isolated from *Sorghum bicolor* in Brazil.	(37)
Klebsiella oxytoca M5a1	Gammaproteobacterium, human pathogen isolated from soil.	(38)
Pseudomonas stuzeri A1501	Gammaproteobacterium, isolated from the rice rhizosphere in southern China.	(39)
Rhodobacter sphaeroides WS8	Alphaproteobacterium, isolated from soil in Ithica, NY.	(40)

211

212 ***in planta* ARA protocol.** *Azorhizobium caulinodans* ORS571 was used as a model strain for
213 validation and optimisation of the *in planta* ARA. Golden promise barley seeds were initially surface
214 sterilised by submersion in 70% ethanol for 2 min and 7% NaOCl for 2 min, then rinsed thoroughly in
215 sterile water and germinated in the dark at room temperature for 2-days on 0.9% water agar. To house
216 the barley seedlings, 130 mL Schott bottles were filled with 50 g of industrial grade sand 15 mL of N-
217 free and C-free rooting solution ($CaCl_2$•$2H_20$ 2.67 mM, KCl 276 µM, $MgSO_4$•$7H_2O$ 2.13 mM, Fe
218 EDTA 26.67 µM, H_3BO_3 93.33 µM, $MnCl_2$•$4H_2O$ 24 µM, $ZnCl_2$ 2.13 µM, Na_2MoO_4•$2H_2O$ 1.33 µM,
219 $CuSO_4$•$5H_2O$ 0.8 µM, KH_2PO_4 1.33 g/L, Na_2HPO_4 1.52 g/L), then autoclaved. One seedling was sewn
220 into each bottle and immediately inoculated with 2 mL of bacterial suspension in UMS. The openings
221 of Schott bottles were subsequently covered with sterile cling film and placed in a growth chamber
222 with a 23 °C 16 h light / 21 °C 8 h dark cycle. At 6-dpi the Schott bottles were placed in a controlled
223 atmosphere cabinet adjusted to 1% O_2 by flushing with N_2 gas, left for one hour and sealed with a
224 rubber septum. To start the assay, ten percent of the headspace atmosphere (16.5 mL of air) was

immediately replaced with C_2H_2 (13 mL) using a 20 mL syringe with needle and the plants were returned to the growth chamber. The evolution of C_2H_4 from C_2H_2 was measured at various timepoints outlined in the results section using GC-MS (Clarus 480 gas chromatograph, PerkinElmer) as previously described (41). Treatments for all experiments were performed with five biological replicates (i.e. five agar slopes of the inoculant and five Schott bottles containing barley seeds).

Recovery of bacteria and estimation of population size. Bacteria were recovered from the root surface and soil of *in planta* ARA systems after 72-h in N-fixing conditions (9-dpi total) by adding 25 mL of PBS to the Schott bottles and vigorously agitating for 30 s. Viable counts were performed by establishing 10-fold serial dilutions of the resulting homogenous bacterial suspension from each Schott bottle and spotting 50 µL aliquots on non-selective agar plates. Colony morphology was examined to confirm that contamination had not occurred, and the total number of cfu present was estimated based on the total volume (60 mL).

Statistical analysis. Total C_2H_4 production was calculated for each timepoint by deriving the fraction of the C_2H_4 peak area compared to C_2H_2, then multiplying this value by the number of C_2H_2 nmoles originally injected into the headspace based on the ideal gas law (5.31×10^5 nmoles C_2H_2). Rates of nitrogenase activity were subsequently calculated between two time points as described in the Results section. All statistical analyses were performed using the agricolae and RStatix packages in R (42) and relevant information regarding each statistical test is provided in the figure captions.

Acknowledgements

TH is the recipient of an 1851 Royal Commission for the Exhibition of 1851 Research Fellowship (RF-2019-100238) and Wolfson College, University of Oxford Junior Research Fellowship. This

work was supported by the Biotechnology and Biological Sciences Research Council [grant numbers BB/L011484/1, BB/T006722/1].

References

1. Santos MS, Nogueira MA, Hungria M. Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. *AMB Express* 2019; **9**: 205-205.
2. Rosenblueth M, Ormeño-Orrillo E, López-López A, Rogel MA, Reyes-Hernández BJ, Martínez-Romero JC *et al.* Nitrogen Fixation in Cereals. *Front Microbiol* 2018; **9**: 1794.
3. Haskett TL, Tkacz A, Poole PS. Engineering rhizobacteria for sustainable agriculture. *ISME J* 2020.
4. Bloch SE, Ryu M-H, Ozaydin B, Broglie R. Harnessing atmospheric nitrogen for cereal crop production. *Curr Opin Biotechnol* 2020; **62**: 181-188.
5. Bueno Batista M, Dixon R. Manipulating nitrogen regulation in diazotrophic bacteria for agronomic benefit. *Biochem Soc Trans* 2019; **47**: 603-614.
6. Li Q, Chen S. Transfer of Nitrogen Fixation (*nif*) Genes to Non-diazotrophic Hosts. *ChemBioChem* 2020; **21**: 1717 –1722
7. Geddes BA, Ryu MH, Mus F, Garcia Costas A, Peters JW, Voigt CA *et al.* Use of plant colonizing bacteria as chassis for transfer of N(2)-fixation to cereals. *Curr Opin Biotechnol* 2015; **32**: 216-222.
8. Ryu MH, Zhang J, Toth T, Khokhani D, Geddes BA, Mus F *et al.* Control of nitrogen fixation in bacteria that associate with cereals. *Nat Microbiol* 2020; **5**: 314-330.
9. Hardy RWF, Holsten RD, Jackson EK, Burns RC. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiol* 1968; **43**: 1185-1207.
10. Doty SL, Sher AW, Fleck ND, Khorasani M, Bumgarner RE, Khan Z *et al.* Variable nitrogen fixation in wild *Populus*. *PLOS ONE* 2016; **11**: e0155979.

11. Van Deynze A, Zamora P, Delaux P-M, Heitmann C, Jayaraman D, Rajasekar S *et al.* Nitrogen fixation in a landrace of maize is supported by a mucilage-associated diazotrophic microbiota. *PLoS Biol* 2018; **16**: e2006352.

12. Herridge DF, Giller KE. Measurement of Nitrogen Fixation. In: Howieson JG, Dilworth, M.J. (eds). *Working with rhizobia*. ACIAR, 2016, pp 187-218.

13. Halbleib CM, Ludden PW. Regulation of biological nitrogen fixation. *The Journal of Nutrition* 2000; **130**: 1081-1084.

14. Ninfa AJ, Jiang P. PII signal transduction proteins: sensors of α-ketoglutarate that regulate nitrogen metabolism. *Curr Opin Microbiol* 2005; **8**: 168-173.

15. Little R, Dixon R. The amino-terminal GAF domain of *Azotobacter vinelandii* NifA binds 2-oxoglutarate to resist inhibition by NifL under nitrogen-limiting conditions. *J Biol Chem* 2003; **278**: 28711-28718.

16. Little R, Reyes-Ramirez F, Zhang Y, van Heeswijk WC, Dixon R. Signal transduction to the *Azotobacter vinelandii* NifL-NifA regulatory system is influenced directly by interaction with 2-oxoglutarate and the PII regulatory protein. *EMBO J* 2000; **19**: 6041-6050.

17. Hurek T, Handley LL, Reinhold-Hurek B, Piché Y. *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. *Mol Plant Microbe Interact* 2002; **15**: 233-242.

18. Bloemberg GV. Microscopic analysis of plant–bacterium interactions using auto fluorescent proteins. *Eur J Plant Pathol* 2007; **119**: 301-309.

19. Egener T, Hurek T, Reinhold-Hurek B. Use of green fluorescent protein to detect expression of *nif* genes of *Azoarcus* sp. BH72, a grass-associated diazotroph, on rice roots. *Mol Plant Microbe Interact* 1998; **11**: 71-75.

20. Egener T, Hurek T, Reinhold-Hurek B. Endophytic expression of *nif* genes of *Azoarcus* sp. strain BH72 in rice roots. *Mol Plant Microbe Interact* 1999; **12**: 813-819.

21. Santos ARS, Etto RM, Furmam RW, Freitas DL, Santos K, Souza EM *et al.* Labeled *Azospirillum brasilense* wild type and excretion-ammonium strains in association with barley roots. *Plant Physiol Biochem* 2017; **118**: 422-426.

22. Roncato-Maccari LDB, Ramos HJO, Pedrosa FO, Alquini Y, Chubatsu LS, Yates MG *et al.* Endophytic *Herbaspirillum seropedicae* expresses *nif* genes in gramineous plants. *FEMS Microbiol Ecol* 2003; **45**: 39-47.

23. Priestley CA, Treharne KJ, Lenz F. Effects of low oxygen on photosynthesis, translocation and growth in green pepper (*Capsicum annuum*). *Ann Bot* 1988; **61**: 159-167.

24. Zabalza A, van Dongen JT, Froehlich A, Oliver SN, Faix B, Gupta KJ *et al.* Regulation of respiration and fermentation to control the plant internal oxygen concentration. *Plant Physiol* 2009; **149**: 1087.

25. Geigenberger P, Fernie AR, Gibon Y, Christ M, Stitt M. Metabolic activity decreases as an adaptive response to low internal oxygen in growing potato tubers. 2000; **381**: 723-740.

26. Geigenberger P. Response of plant metabolism to too little oxygen. *Curr Opin Plant Biol* 2003; **6**: 247-256.

27. Igamberdiev AU, Stasolla C, Hill RD. Low oxygen stress, nonsymbiotic hemoglobins, NO, and programmed cell death. In: van Dongen JT, Licausi F (eds). *Low-Oxygen Stress in Plants: Oxygen Sensing and Adaptive Responses to Hypoxia*. Springer Vienna: Vienna, 2014, pp 41-58.

28. Mascher M, Gundlach H, Himmelbach A, Beier S, Twardziok SO, Wicker T *et al.* A chromosome conformation capture ordered sequence of the barley genome. *Nature* 2017; **544**: 427-433.

29. Grosskopf T, Soyer OS. Synthetic microbial communities. *Curr Opin Microbiol* 2014; **18**: 72-77.

30. Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* 1951; **62**: 293-300.

31. Beringer JE. R factor transfer in *Rhizobium leguminosarum*. *Microbiology* 1974; **84**: 188-198.

32. Poole PS, Schofield NA, Reid CJ, Drew EM, Walshaw DL. Identification of chromosomal genes located downstream of *dctD* that affect the requirement for calcium and the lipopolysaccharide layer of *Rhizobium leguminosarum*. *Microbiology* 1994; **140 (Pt 10)**: 2797-2809.

33. Chen MH, Sheu SY, James EK, Young CC, Chen WM. *Azoarcus olearius* sp. nov., a nitrogen-fixing bacterium isolated from oil-contaminated soil. *Int J Syst Evol Microbiol* 2013; **63**: 3755-3761.

34. Dreyfus B, L. GJ, Gillis M. Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. *Int J Syst Evol Microbiol* 1988; **38**: 89-98.

35. Tarrand JJ, Krieg NR, Döbereiner J. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Can J Microbiol* 1978; **24**: 967-980.

36. Doty SL, Dosher MR, Singleton GL, Moore AL, Aken Bv, Stettler RF *et al.* Identification of an endophytic Rhizobium in stems of *Populus*. *Symbiosis* 2005; **39**: 27-35.

37. Baldani JI, Pot B, Kirchhof G, Falsen E, Baldani VL, Olivares FL *et al.* Emended description of *Herbaspirillum*; inclusion of *Pseudomonas rubrisubalbicans*, a milk plant pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov.; and classification of a group of clinical isolates (EF group 1) as *Herbaspirillum* species 3. *Int J Syst Evol Microbiol* 1996; **46**: 802-810.

38. Hamilton PB, Wilson PW. Nitrogen fixation by *Aerobacter aerogenes*. *Annales Academiæ Scientiarum Fennicæ* 1955; **60**: 139-150.

39. Qui YS, Zhou SP, Mo XZ. Study of nitrogen fixing bacteria associated with rice root. 1: isolation and identification of organisms. *Acta Microbiol Sinica* 1981; **21**: 468–472.

40. Clayton RK, Clayton BJ. Relations between pigments and proteins in the photosynthetic membranes of *Rhodopseudomonas spheroides*. *Biochimica et Biophysica Acta* 1972; **283**: 492-504.

41. Trinick MJ, Dilworth MJ, Grounds M. Factors affecting the reduction of acetylene by root nodules of *Lupinus* species. *New Phytol* 1976; **77**: 359-370.

42. R Core Team. R: A language and environment for statistical computing 2021: Vienna, Austria.http://www.R-project.org/

359

360