Research

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A genome-scale metabolic reconstruction of soybean and *Bradyrhizobium diazoefficiens* reveals the cost–benefit of nitrogen fixation

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Summary

- Nitrogen-fixing symbioses allow legumes to thrive in nitrogen-poor soils at the cost of diverting some photoassimilate to their microsymbionts. Effort is being made to bioengineer nitrogen fixation into nonleguminous crops. This requires a quantitative understanding of its energetic costs and the links between metabolic variations and symbiotic efficiency.
- A whole-plant metabolic model for soybean (*Glycine max*) with its associated microsymbiont *Bradyrhizobium diazoefficiens* was developed and applied to predict the cost–benefit of nitrogen fixation with varying soil nitrogen availability.
- The model predicted a nitrogen-fixation cost of *c.* 4.13 g C g^{-1} N, which when implemented into a crop scale model, translated to a grain yield reduction of 27% compared with a non-nodulating plant receiving its nitrogen from the soil. Considering the lower nitrogen content of cereals, the yield cost to a hypothetical N-fixing cereal is predicted to be less than half that of soybean. Soybean growth was predicted to be *c.* 5% greater when the nodule nitrogen export products were amides versus ureides.
- This is the first metabolic reconstruction in a tropical crop species that simulates the entire plant and nodule metabolism. Going forward, this model will serve as a tool to investigate carbon use efficiency and key mechanisms within N-fixing symbiosis in a tropical species forming determinate nodules.

Introduction

Nitrogen (N) is often limiting for crop growth since it is a key component of proteins, nucleic acids, chlorophylls, and many other molecules of primary and secondary metabolism (Hodges, 2002). Globally, agriculture uses 111 Tg N fertilizer yearly to support crop production and food security (FAO, 2019). Over application of N fertilizer results in N leaching into water bodies, and production of nitrogenous greenhouse gasses that are 300-times more powerful than CO_2 (Galloway *et al.*, 2008; Rockström *et al.*, 2009; Good, 2018). N fertilizer production is the largest consumer of fossil fuels in agriculture and by 2050 is expected to represent 2% of global energy consumption (Glendining *et al.*, 2009). Alternatively, diazotrophic bacteria biologically fix atmospheric N_2 via nitrogenase (Hoffman *et al.*, 2014). Plants benefit from this by absorbing N either from the secretions of free-living bacteria in the soil or, in the case of legumes, via endosymbiosis with bacteria known as rhizobia. N symbiosis provides plants a source of bio-available N, reducing the need for N fertilizers. As of 2021, almost 1 billion people are estimated to have insufficient food, with over a quarter of these in sub-Saharan Africa (Long *et al.*, 2022). Bioengineering N symbioses into nonleguminous crops has the potential to increase yields in parts of the world where costs, or lack of transport infrastructure, prevent access to fertilizers (Oldroyd & Dixon, 2014; Rogers & Oldroyd, 2014; Oldroyd & Leyser, 2020), while substantially reducing pollution in wealthier agricultural economies. Although it is unlikely that engineered N symbiosis could entirely replace the need for fertilizers, it has been estimated that engineering maize capable of fixing 50 kg ha^{-1} of N would double yield in sub-Saharan Africa from 1.58 to 3.32 Mg ha^{-1} (Folberth *et al.*, 2013; Rogers & Oldroyd, 2014).

Endosymbiotic N-fixing bacteria colonize root cells and in the case of rhizobia are housed in plant-membrane bound compartments known as symbiosomes in nodules formed in response to infection (Ledermann *et al.*, 2021). Ammonia is excreted from the symbiosome, which is used to produce amino acids or ureides in exchange for carbon (C) compounds, most commonly malate, succinate, and fumarate (Lodwig & Poole, 2003). N fixation is costly, using 16 moles of ATP to produce 2 moles of ammonia.

This accounts for most of the energy consumed within the nodule (Streeter, 1981; Lodwig & Poole, 2003; Valentine *et al.*, 2010). Additional energy is used to produce and maintain the nodule, and to maintain low oxygen conditions within the bacteroid since nitrogenase is oxygen sensitive (Rutten & Poole, 2019). A key question then is how much productivity does N-fixation cost, and under what conditions of soil N will it be beneficial and when would it be detrimental? This requires a quantification of the amount of C lost per N fixed.

The earliest measurements of N-fixation costs varied greatly. These inconsistencies were caused by mishandling of the oxygen diffusion barrier via either acetylene reduction assays, removal of nodules, or disturbing the roots, slowing O_2 diffusion across the nodule cortex (Schubert, 1982; Minchin *et al.*, 1983, 1986; Witty *et al.*, 1984; Sheehy & Phillips, 1987), as well as metabolic variation across the various plant and bacterial partners (Minchin & Witty, 2005). Estimations of ATP requirements for the reactions occurring within a nodule give a range of 2.78–4.81 g C g^{-1} N (Minchin & Witty, 2005). However, understanding the full cost requires a consideration of all plant and microbe metabolism, for specific plant–microbe interactions. Such analyses are now enabled by integration of transcriptomics and flux balance analysis (FBA) metabolic models (Pfau *et al.*, 2018; diCenzo *et al.*, 2020).

Many different assumptions have been used in modelling N fixation in plants, some estimating N-fixation rate from plant N demand/uptake (Cabelguenne *et al.*, 1999; Fitton *et al.*, 2019) or the mass of different plant compartments, that is root, nodule, or aboveground biomass (Sinclair, 1986; Thornley *et al.*, 1995; Wu & McGechan, 1999; Boote *et al.*, 2002; Robertson *et al.*, 2002; Soussana *et al.*, 2002). Alternatively, FBA models forego such assumptions by directly simulating fluxes through the complete metabolic network built from genome annotations. Metabolic fluxes are obtained by optimizing an objective function, for example maximizing growth in the presence of specific metabolic constraints, such as the rate of ammonium and photon uptake. This provides a global view of the metabolism of an organism under steady-state conditions without specific information on the kinetic parameters of each enzyme. FBA model reconstructions have been built for several N-fixing legume symbionts, including *Sinorhizobium fredii* (Contador *et al.*, 2020), *Bradyrhizobium diazoefficiens* (Yang *et al.*, 2017), *Rhizobium etli* (Resendis-Antonio *et al.*, 2007), *Rhizobium leguminosarum* (Schulte *et al.*, 2021, 2022), and *Sinorhizobium meliloti* (Zhao *et al.*, 2012; diCenzo *et al.*, 2016, 2018, 2020). Most studies investigate symbiotic N fixation by performing simulations with isolated bacteroids, investigating individual processes that would typically be controlled by the plant. Current whole plant models for simulating symbiotic N fixation are limited to *M. truncatula* (Pfau *et al.*, 2018; diCenzo *et al.*, 2020), a model temperate species that forms indeterminate nodules and exports N from nodules in the form of amides (Vance, 2000). While the *M. truncatula-S. meliloti* mutualism is a good model for temperate conditions, these models cannot capture the large metabolic variation observed between various host plant–bacteria interactions, necessitating a need for nodulated whole-plant models of more species.

Soybean is an exceptionally well-studied and highly productive sub-tropical crop, which forms determinate nodules that host bradyrhizobia. The nodule metabolism of tropical and temperate legumes differ in the form in which N is exported from the nodule. Tropical legumes, including soybean, more commonly export the ureides allantoin and allantoic acid, rather than the amides glutamine and asparagine, as in temperate legumes (Serraj *et al.*, 1999). It has been hypothesized that ureide export consumes less carbon than amide exported for two reasons (Schubert, 1986; Atkins, 1991; Todd *et al.*, 2006). First, synthesis rates are thought to be higher for ureides, (Schubert, 1986) and second, ureides have a lower C/N ratio than amides, 1 : 1 for ureides vs 2 : 1 for asparagine (Welder, 2014). This suggests less photosynthetically assimilated carbon will be needed to acquire N for ureide producing legumes (Schubert, 1986; Atkins, 1991; Todd *et al.*, 2006). In addition, Todd *et al.* (2006) estimated that five ATP are required to produce one molecule of allantoin or allantoate compared with 12 ATP for asparagine production.

Here, we present a metabolic model of a nodulated soybean plant hosting *Bradyrhizobium diazoefficiens*. The model is used to investigate the effect of N symbiosis on growth at the whole-plant scale and its predictions are integrated with a crop scale model, Soybean-BioCro (Matthews *et al.*, 2022), to predict the cost of N fixation on soybean yield. This is applied to address the following questions. (1) What is the carbon cost (g C g^{-1} N) of N fixation in soybean? (2) Is ureide export from the nodule more costly than amide export in soybean? (3) What effect does N fixation have on soybean growth and yield?

Materials and Methods

Model description

A FBA model describing the metabolism of a soybean (*Glycine max* (L.) Merr) plant nodulated by the rhizobium *B. diazoefficiens* (Delamuta *et al.*, 2013) USDA 110 was built from a list of reactions, represented as a stoichiometric matrix identifying the metabolites produced and consumed by each individual reaction. The model was initially built from a set of reactions generated by Moreira *et al.* (2019), which is a FBA model of a soybean seedling, and integrated with the previously reported *B. diazoefficiens* USDA 110 FBA model (Yang *et al.*, 2017). Our model encompasses the reactions occurring within the shoot (leaf and stem), the root, and the nodule tissues, where each organ represents an individual compartment. Each plant compartment (e.g. shoot, root, and nodule) contains the subcellular organelle compartments cytosol (c), mitochondria (m), peroxisome (x), extracellular (e), and plastid (p). The N-fixing bacteroids are also simulated as an organelle compartment within the nodule (Fig. 1) and contain the subcellular compartments cytosol (c) and extracellular (e). The shoot and root organs each contain biomass reactions that determine their growth (see Supporting Information Dataset S1), with these reactions accounting for 72 and 67 biomass metabolites, respectively. The metabolites and associated stoichiometries are based on biomass compositions measured for

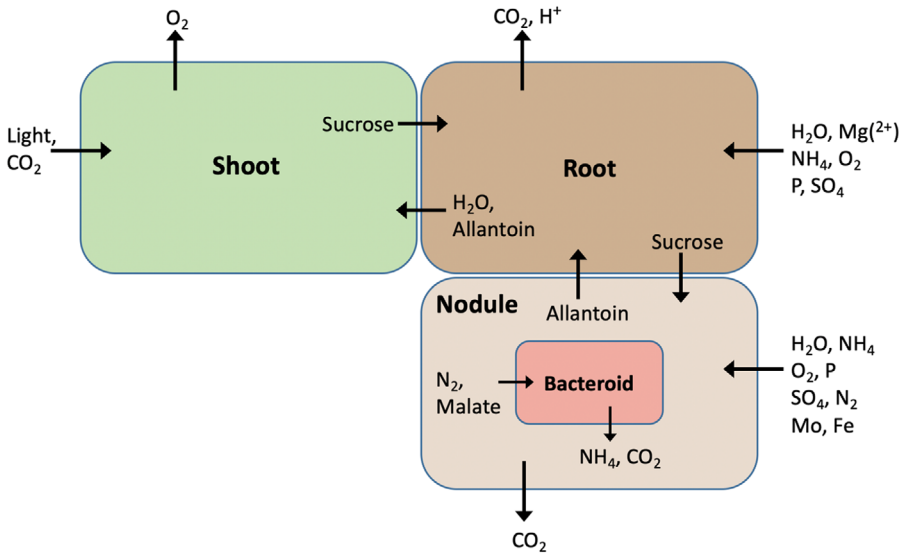

Fig. 1 Visualization of the net exchanges of major metabolites between the compartments within the soybean model. The model contains shoot, root, and nodule compartments. The transport of metabolites (black arrows) is allowed between compartments and with the external environment.

the leaf, root, and stem (see Dataset S2), and include metabolites such as cell wall components (i.e. hemicellulose, cellulose, pectins, and lignins), proteins, lipids, nucleic acids, chlorophyll, soluble metabolites, and starch. The model includes specialized secondary metabolism required for constructing biomass metabolites (see Dataset S2) and any that were identified as highly expressed from RNA-Seq data. The nodule organ is specific for N fixation and does not contain a biomass reaction; instead, growth of new determinate nodule tissue is modelled as a root sink reaction. Transfer of metabolites between plant organs is simulated via transport reactions. The exchange of metabolites across the peribacteroid membrane is simulated with transport reactions between the nodule cytosol and the bacteroid extracellular compartment (Fig. 1). The uptake and release of metabolites between the plant and the external environment are simulated by exchange reactions. Where possible, reaction and metabolite IDs correspond to the MetaNetX database (Moretti *et al.*, 2021), with additional IDs assigned to each feature from the KEGG, BiGG, MetaCyc, ChEBI, and Rhea databases (Hastings *et al.*, 2016; King *et al.*, 2016; Caspi *et al.*, 2020; Bansal *et al.*, 2022; Kanehisa *et al.*, 2023). A full list of reactions included in the model is provided in Dataset S1.

Total plant biomass was assumed to be the sum of the growth of the shoot, root, and nodule, where nodule mass is assumed to account for 2% of total plant mass (Chen *et al.*, 2014; Moretti *et al.*, 2018; diCenzo *et al.*, 2020) and the root : shoot ratio is 0.17 (Ordóñez *et al.*, 2020). The FBA model was solved by linear programming using an algorithm to optimize the rates of all model reactions in order to maximize total plant biomass production, with the assumption that all metabolites are at a steady-state concentration (Orth *et al.*, 2010). The FBA model is static, predicting metabolism at one time point, and was used to predict whole-plant relative growth rate (RGR; g g^{-1} DW d^{-1}). The simulated costs of N fixation on RGR were then incorporated into a separate multiscale crop model, Soybean-BioCro (Matthews *et al.*, 2022), to predict yield (Fig. 2).

Whole-plant FBA model reconstruction

The nodulated soybean FBA model was built from the unification of two previously published genome-scale reconstructions of soybean (Moreira *et al.*, 2019) and *B. diazoefficiens* USDA 110 (Yang *et al.*, 2017) metabolism. Mass and charge balancing, gap filling, and validation were performed as part of the original reconstruction processes (Yang *et al.*, 2017; Moreira *et al.*, 2019), with mass and charge balancing updated following construction of the integrated model. Integration of the models was accomplished using MATLAB code adapted from diCenzo *et al.* (2020) and functions from the COBRA TOOLBOX (Schellenberger *et al.*, 2011). Briefly, the soybean model was duplicated with two copies representing the shoot and root organs, and specific metabolites were allowed to move between organs. Then, the reaction space of the shoot and root organs were constrained using FASTCORE (Vlassis *et al.*, 2014; Pacheco & Sauter, 2018) guided by previously published soybean shoot- and root-specific RNA-Seq data (Severin *et al.*, 2010). This 'plant' model was then combined with a nodule model that included the *B. diazoefficiens* model embedded within the nodule model. The reaction space of the nodule was then constrained using GIMME (Becker & Palsson, 2008) and published RNA-Seq datasets previously generated for soybean nodule cells and *B. diazoefficiens* bacteroids (see Methods S1, page 3; Delmotte *et al.*, 2010; Severin *et al.*, 2010). The full code for model reconstruction can be found on GitHub (https://github.com/bop15bh/SoybeanFBA), while a detailed description of the model reconstruction process, as well as reaction and metabolite labelling conventions, are provided in the Methods S1.

Adding amide export to the nodulated soybean model

To determine how the use of ureides vs amides as the nodule N export product impacts the carbon cost of symbiotic N fixation, a modified version of the nodulated soybean model was

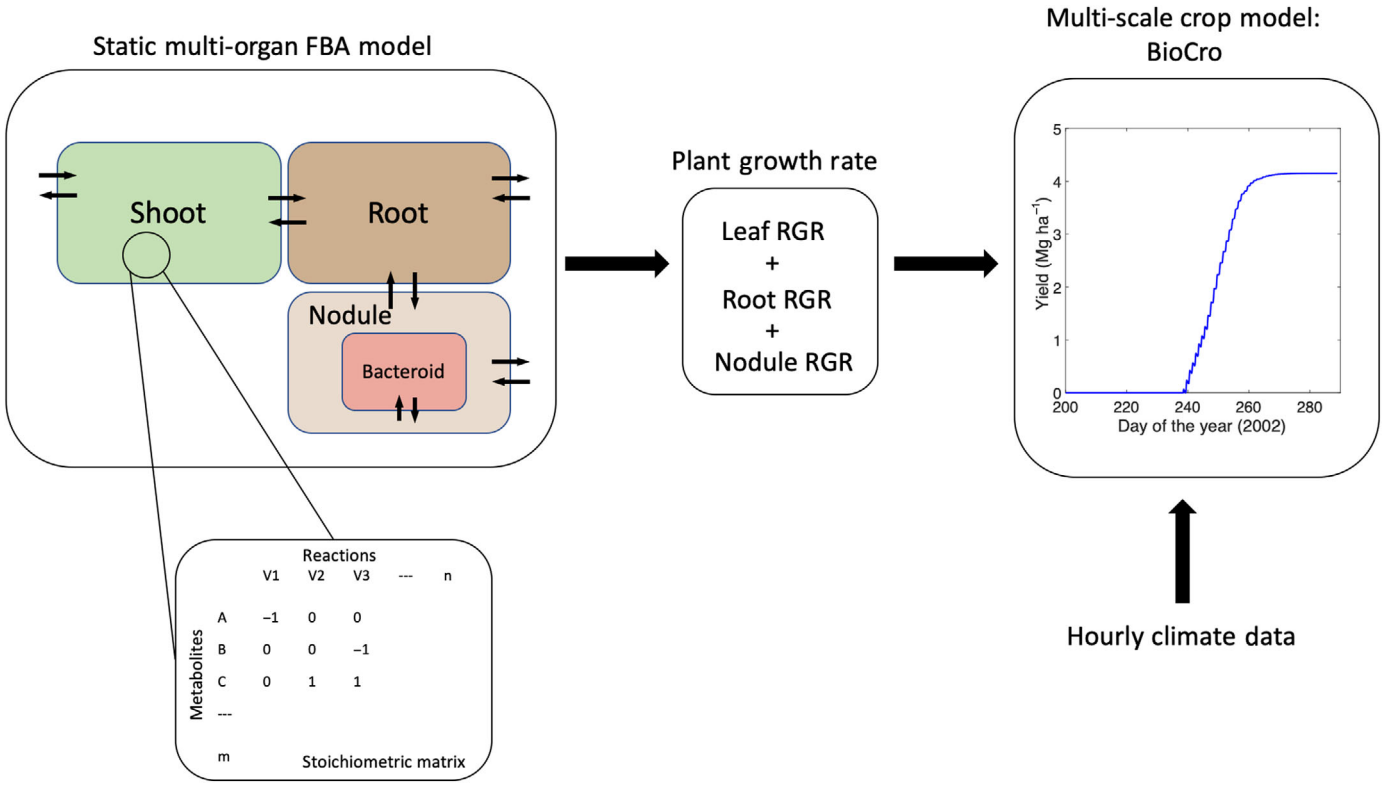

Fig. 2 Unification of the static flux balance analysis (FBA) model of N fixation in soybean with a crop level simulation. The FBA model predicts plant relative growth rate (RGR) and costs in RGR when only biological fixation is the only source of N. This cost is then input to Soybean-BioCro by altering allocation along with climate data for the Illinois 2002 and 2005 growing season. Arrows show direction of model input.

constructed. To do this, a second model was generated using a model reconstruction process similar to that described above and in the Methods S1. The sole difference was that before constraining the nodule reaction space, the reactions for the transport of allantoate and allantoin from the nodule to the root were removed and replaced with reactions for the transport of glutamine and asparagine. Then, all reactions present in the new model that were both nonessential for optimal growth rate and were absent from the original model were identified and removed. Subsequently, an updated nodulated soybean model was constructed by taking the union of the reaction space of the new and original models; the resulting model was capable of exporting N from the nodule in the form of ureides or amides, and the optimized flux determines which product is preferred.

Metabolic modelling software and solvers

Flux balance analysis simulations, and most of the model integration and manipulations, were performed in MATLAB R2020b (www.mathworks.com) using libSBML v.5.17 (Bornstein *et al.*, 2008), and scripts from the COBRA TOOLBOX v.3.0.6 (Schellenberger *et al.*, 2011), the TIGER TOOLBOX v.1.3.1 (Jensen *et al.*, 2011), FASTCORE v.1.0 (Vlassis *et al.*, 2014; Pacheco & Sauter, 2018), and the TN-CORE TOOLBOX v.2.3 (diCenzo *et al.*, 2019). The GLPK SOLVER v.5.0 (www.gnu.org/software/glpk).

The exception to the above was when running GIMME as well as all downstream model construction steps. These steps were performed in MATLAB R2019a using the ILOG CPLEX STUDIO 12.9.0 solver, SBMLTOOLBOX v.4.1.0 (Keating *et al.*, 2006), libSBML v.5.17, and scripts from the COBRA TOOLBOX v.3.1, the TIGER TOOLBOX v.1.3.1, FASTCORE v.1.0, and the TN-CORE TOOLBOX v.2.3.

Plant level simulation

When performing FBA simulations, and unless stated otherwise, most reactions were unconstrained with upper and lower bounds of 1000 μmol g^{-1} DW h^{-1}, if reversible, or with either the upper or lower bound set to 0 μmol g^{-1} DW h^{-1} if irreversible. A notable exception was the photon uptake reaction in the shoot compartment, which had an upper bound of 2920 μmol g^{-1} DW h^{-1} based on Farquhar *et al.* (1980). Moreover, most reactions transferring metabolites from the nodule plant cells to the bacteroids had upper bounds of 5 or 10 nmol g^{-1} DW h^{-1} to ensure bacteroids used C_4-dicarboxylates as the primary source of carbon, as used elsewhere (diCenzo *et al.*, 2020). In addition, nongrowth-associated maintenance costs were incorporated into the model by setting the lower bounds of the shoot, root, nodule, and bacteroid ATP hydrolysis reactions to 92.22, 18.89, 0.83, and 6.30 nmol g^{-1} DW h^{-1}, respectively, as in diCenzo *et al.* (2020).

The growth cost of N fixation was predicted with different levels of soil N availability. This was achieved by setting the upper bound of the ammonium uptake reaction to zero when simulating no soil N and increasing it to simulate successively higher soil N availability. Ammonium uptake is a closer estimation of the effects of N fixation on growth as it is less costly to the plant than nitrate, which must first be reduced to ammonium for assimilation. Cereals have a much lower N content per plant, and so N requirement is significantly lower than for legumes. For example, average N content of the whole plant (grain and stover) at harvest is 3.1% for soybean compared with 1.4% for maize (Reddy *et al.*, 2013; Tamagno *et al.*, 2017). The FBA predicted growth cost of N fixation was scaled accordingly to predict the potential cost of N fixation for engineered maize, assuming that a plant that has 55% less N content would also have a similar reduction in N-fixation rate per unit plant mass.

Flux variability analysis (FVA) was used to identify the range of possible nodule CO_2 fluxes that generate model solutions that are ≥ 99% of the maximal soybean RGR in the absence of soil N (Mahadevan & Schilling, 2003). Doing so involved using linear programming to determine all possible metabolic fluxes for a specified list of reactions, nodule CO_2 flux in this case, and identify which generate a RGR within the desired tolerance, that is ≥ 99% of RGR. This was performed using the COBRA TOOLBOX in MATLAB.

Calculating the carbon costs of N fixation

The carbon cost of symbiotic N fixation (g C g^{-1} N) was calculated based on optimal fluxes from the solved FBA model. This was done in two ways. First, by measuring the rate of ammonium production via nitrogenase (V_n) against the rate of CO_2 efflux out of the nodule (V_c):

$$\text{C : N ratio} = \frac{V_c}{2V_n} \quad \text{Eqn 1}$$

Second, by measuring the difference in carbon flux into the nodule minus the flux of carbon from the nodule to the root over the flux of nitrogen leaving the nodule minus the flux of nitrogen (excluding N_2) entering the nodule:

$$\text{C : N ratio} = \frac{C_{in} - C_{out}}{N_{out} - N_{in}} \quad \text{Eqn 2}$$

where fluxes are scaled by the number of carbon or nitrogen atoms within the metabolite.

Crop level simulation

Soybean-BioCro (Matthews *et al.*, 2022) was used to predict the effect of N fixation on yield at the crop level. Soybean-BioCro is the soybean specific version of BioCro (Lochocki *et al.*, 2022), a crop growth model that scales from photosynthetic processes to whole crop growth using hourly climate data including solar radiation, air temperature, wind speed, precipitation, and humidity. Soybean-BioCro was calibrated with 2002 and 2005 climate and biomass data from the SoyFace Facility in Urbana, Illinois, USA (Matthews *et al.*, 2022). Soybean-BioCro was then validated against soybean biomass measurements from the 2004 and 2006 growing season, and from soybeans grown in elevated atmospheric CO_2 during the 2002 and 2004–2006 growing seasons (Morgan *et al.*, 2005; Bishop *et al.*, 2015; Matthews *et al.*, 2022). The RMSEs of the predicted vs average leaf, stem, and pod biomasses measured over the growing seasons ranged from 0.4 to 1.0 Mg ha^{-1}. The final predicted pod biomasses were within one standard deviation of the mean of experimental measurements for all but 1 yr of the validation cases (Matthews *et al.*, 2022).

The predicted costs of N fixation from the FBA model (see 'Plant level simulation' in the Materials and Methods section) were incorporated into Soybean-BioCro by diverting the proportion of photosynthate predicted by FBA as required for N fixation, away from the growth and maintenance of the rest of the plant.

Value cost ratio (VCR) was used to estimate the profitability of crop inoculum treatment based on yield and current prices.

$$\text{VCR} = \frac{(Y_t - Y_c)P_g}{P_t} \quad \text{Eqn 3}$$

where Y_t is the yield with inoculant or fertilizer (Mg ha^{-1}), Y_c is the control yield without any treatments (Mg ha^{-1}), P_g is the unit price of soybean seed (\$ kg^{-1}), and P_t is the unit price of inoculant or fertilizer (\$ ha^{-1}). A VCR larger than 3 is considered to be profitable (Thompson, 1991).

Results

Reconstruction of a metabolic model for nodulated soybean

A nodulated soybean metabolic model was generated by combining existing soybean and *B. diazoefficiens* USDA 110 models (Yang *et al.*, 2017; Moreira *et al.*, 2019) using the framework described by diCenzo *et al.* (2020). The resulting model consists of shoot, root, and nodule tissues, with the nodule tissue dedicated to N fixation and the growth of new nodule tissue represented as a root sink reaction. Overall, the model contains 2258 reactions (830 in the shoot, 803 in the root, 265 in the nodule plant compartments, and 134 in the bacteroids) associated with 2943 nonredundant genes (132 bacterial genes and 2811 plant genes of which 100 are associated with nodule reactions).

When FBA was used to optimize the metabolic model to maximize RGR in the absence of any soil N, the nodule produced nearly 7 μmol g^{-1} DW h^{-1} of ammonium while receiving *c.* 3.3 μmol g^{-1} DW h^{-1} of sucrose from the rest of the plant (Fig. 3). The majority of the sucrose was metabolized into malate, which was provided to the bacteroid together with molybdate, homocitrate, sulfate, and iron (Fig. 3). These compounds are required for synthesis of the FeMo cofactor of nitrogenase, N-fixation and bacteroid maintenance (Fig. 3). In turn, the bacteroid secreted water, CO_2, and fixed nitrogen in the form of ammonia.

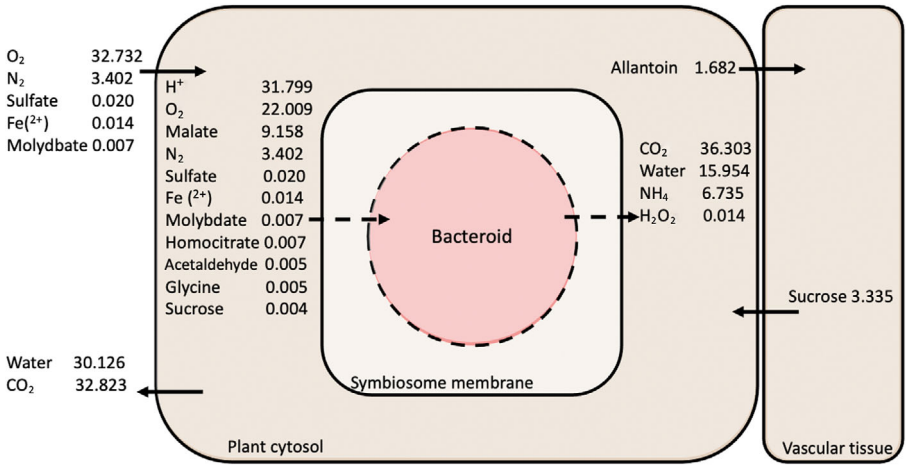

Fig. 3 Net fluxes (μmol g^{-1} DW h^{-1}) of metabolites between compartments within the soybean nodule optimized to deliver the maximum plant relative growth rate. Arrows show the net direction of fluxes. Solid arrows show transfer between the nodule and the root or the external environment, whereas dashed arrows show transfer between the nodule plant cells and bacteroids.

The effect of N fixation on plant growth

The cost of N fixation on growth was determined by simulating growth with and without soil nitrogen. The simulations assume that N fixation is facultative, that is N fixation is used only when there is insufficient soil N available, and that as the soil ammonium availability increases, less N is fixed by the nodules. As expected, increasing the proportion of N supplied from the soil vs from N fixation increased RGR (Fig. 4a). In the absence of any available soil N, RGR was 0.055 g g^{-1} DW d^{-1} with a N fixation rate of *c.* 6.8 μmol NH_4 g^{-1} DW h^{-1}. When all N was provided by the soil, with zero N fixation and no nodule formation assumed, the RGR was 0.079 g g^{-1} DW d^{-1}. Therefore, the model predicts that symbiotic N fixation reduces the potential RGR of soybean by *c.* 30% compared with a plant that obtains all of its N from ammonium in the soil. Seventy-eight percent of this RGR difference was associated with the direct costs of N fixation, while the remaining 22% was due to the costs of nodule growth and maintenance (Fig. 4a). Soybean has a high N demand compared with cereals, and thus, the costs of N fixation are likely to be lower for cereals. Indeed, the FBA model predicts that if the N requirements of soybean were equivalent to that of maize, then N fixation would only cost *c.* 14% of the RGR (Fig. 4a).

We additionally tested the impact of increasing CO_2 uptake in the model when relying on N fixation as a source of nitrogen. Raising CO_2 uptake to 183 μmol g^{-1} DW h^{-1} (a 30% increase compared with the default model parameters) resulted in a RGR equivalent to that obtained when all N was provided as soil ammonium (see Methods S1). This result is consistent with the potential for soybean to minimize the metabolic costs of symbiotic N fixation through increasing its photosynthetic rate.

Carbon cost of nitrogen fixation

The FBA model yields a carbon cost of symbiotic N fixation of 4.13 g C g^{-1} N based on the net rate of nodule CO_2 efflux (1) and 4.24 g C g^{-1} N based on the exchange of resources into and out of the nodule (2) (Fig. 3). To test the sensitivity of the predicted carbon cost to rates of photosynthesis, model parameters were modified such that shoot CO_2 uptake was the growth limiting reaction, and the rate of CO_2 uptake was varied. Results indicated that while the carbon costs increased slightly as CO_2 uptake

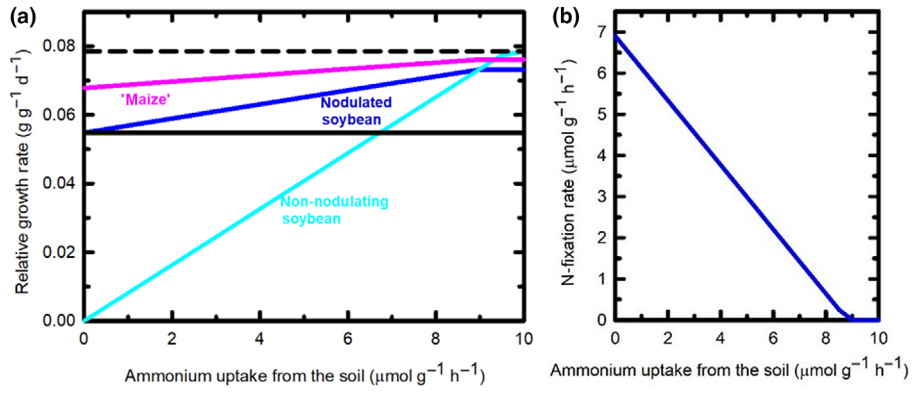

Fig. 4 (a) Increasing soil ammonium uptake rate increases soybean growth rate. Black line represents the growth rate of a nodulated plant when no ammonium is available in the soil (0.055 g g^{-1} DW d^{-1}), and the black dashed line represents growth rate of a non-nodulated plant with a nongrowth rate limiting supply of soil ammonium (0.079 g g^{-1} DW d^{-1}). The blue line shows nodulated soybean growth while the cyan line shows growth of a non-nodulating soybean. The purple line represents a scaled relative growth rate of soybean, but with the lower N requirement of maize, assuming a 55% lower C : N ratio of the plant at harvest. (b) Increasing the soil ammonium uptake rate vs N-fixation rate.

(and thus photosynthesis) was reduced, the predicted costs remained within a narrow range of 4.1–4.8 g C g^{-1} N until CO_2 uptake became highly restricted (see Methods S1; Fig. 1). Furthermore, due to the static nature of FBA modelling, a root : shoot ratio of 0.17 was used in the model. Ordóñez *et al.* (2020) observed that this ratio varies between 0.09 and 0.26. A sensitivity analysis was therefore performed, in which the root : shoot ratio was varied between 0.09 and 0.26 and the impact on carbon costs and RGR was monitored. Changing the root : shoot ratio had little impact on the model predictions; the carbon costs were estimated to be between 4.13 and 4.15 g C g^{-1} N, while the RGR ranged between 0.054 and 0.056 g g^{-1} DW d^{-1} for nodulated soybean (see Methods S1; Fig. 2).

We next investigated how carbon costs are influenced by the form in which N is exported from the nodule. Contrary to expectations, replacing ureide export with amide export in the form of asparagine and glutamine increased RGR by 5% to 0.058 g g^{-1} DW d^{-1} and reduced the carbon cost of N fixation by 16% to 3.47 g C g^{-1} N (1) (Table 1). The reduced carbon cost resulted from a lower nodule net CO_2 efflux when compared to ureide export (Table 1). One limitation of FBA is that it reports only one of possibly many optimal flux solutions. To further investigate the impact of amide vs ureide export on nodule net CO_2 efflux, FVA was performed to identify the range of nodule respiration flux rates compatible with maintaining ≥ 99% of the maximal soybean RGR for both the ureide and the amide model (Table 1). The range of carbon costs of N fixation for the amide model was larger compared with the ureide producing model and overlapped the majority of the range for the ureide model (Fig. 5). Overall, these results show that carbon cost of symbiotic N fixation can be higher in ureide exporting nodules compared with amide exporting nodules but may not always be the case, depending on other metabolic fluxes within the model.

The model contains separate reactions for the export of allantoin and allantoate to the root. In FBA simulations, all N exported from the model occurred through allantoin. To explore whether this was because allantoin was metabolically favorable compared with allantoate as the N export product, the nodule allantoin export reaction was removed from the model, thereby forcing the use of allantoate. Using allantoate as the nitrogen export product instead of allantoin had no effect on RGR and little impact on the carbon cost of nitrogen fixation (Fig. 5).

Table 1 Soybean model output comparing the use of ureides (allantoin and allantoate) and amides (asparagine and glutamine) as the nitrogen export product for transfer of nitrogen from the nodule to the plant.

Model output	Ureide model	Amide model
Relative growth rate (g g^{-1} DW d^{-1})	0.055	0.058
Carbon cost of N fixed (g C g^{-1} N)	4.13	3.47
N fixation (μmol g^{-1} DW h^{-1})	6.81	7.20
Nodule net CO_2 efflux (μmol g^{-1} DW h^{-1})	32.815	29.128

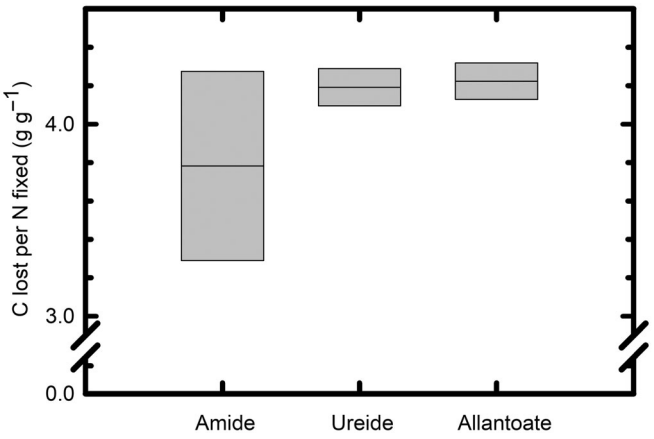

Fig. 5 Flux variability analysis comparing the carbon cost of nitrogen fixation (g C g^{-1} N) for the soybean amide producing model (both asparagine and glutamine) and for the soybean ureide producing model using either allantoin or allantoate, or just allantoate, while maintaining ≥ 99% of the maximal relative growth rate (RGR), which was 0.058 g g^{-1} DW d^{-1} for the amide and 0.055 g g^{-1} DW d^{-1} for the ureide model. Horizontal lines represent midway values in the range of flux variability. Simulations were performed assuming zero availability of soil nitrogen.

Crop yield predictions

The FBA model predicted that provision of all plant nitrogen via symbiotic N fixation decreased RGR by *c.* 30% (*c.* 28.8% to *c.* 31.5% depending on root : shoot ratio) relative to a plant that obtained all of its required N from the soil in the form of ammonium. To estimate the impact on the yield of soybean over a growing season, this reduction was integrated into the crop growth model Soybean-BioCro. Soybean-BioCro predicted a yield of 4.15 Mg ha^{-1} for a non-nodulating plant fulfilling its N requirement entirely through uptake of ammonium from the soil. This yield was reduced by *c.* 27% to 2.99–3.11 Mg ha^{-1} (depending on root : shoot ratio) when all of the plant nitrogen was provided by symbiotic N fixation in the nodules (Fig. 6). To estimate the profitability of fertilizer vs inoculant application, the VCR was used (see the Materials and Methods section). Based on data from small holder farms located in multiple locations in Sub-Saharan Africa (Ethiopia, Ghana, Malawi, Uganda, Mozambique and Zambia), the average yield for uninoculated soybean that had not received N fertilizer is 2.066 Mg ha^{-1} (see Dataset S3). Using this average and a unit price of soybean of 0.4 $ kg^{-1} (see Dataset S3), and the yield predictions for the default root : shoot ratio, the VCR for the predicted yield based on the nodulated soybean FBA-Biocro is between 10.3 (when inoculant is 38 $ ha^{-1}; Ulzen, 2019) and 28.1 (when inoculant is 14 $ ha^{-1}; see Dataset S3). The VCR for fertilized soybean is calculated to be between 3.17 (when urea is 1.51 $ kg^{-1}; Bonilla Cedrez *et al.*, 2020) and 5.99 (when urea is 0.8 $ kg^{-1}; Bonilla Cedrez *et al.*, 2020) based on a treatment of 174 kg ha^{-1} of N in the form of urea, to support a higher yield (Hungria *et al.*, 2006). Based on these values, nodulated soybean shows a much higher profitability (i.e. return in investment) than fertilized soybean even when the price of fertilizer is low and the cost of inoculant is high.

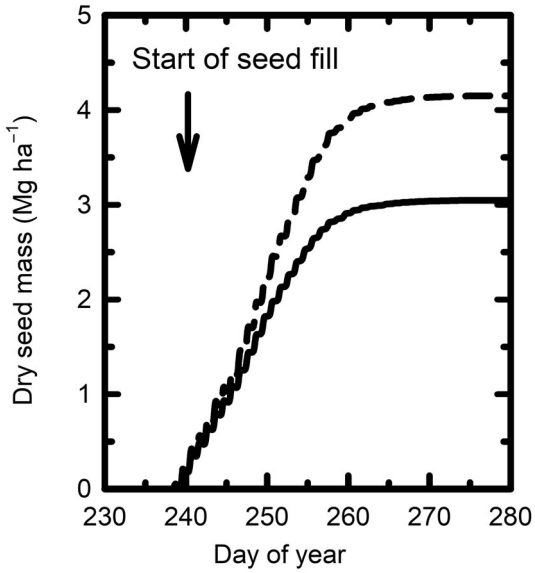

Fig. 6 Soybean grain yield (Mg ha^{-1}) simulated with (solid line) and without (dashed line) a 30.2% reduction in the amount of photosynthate partitioned to the different organs. Simulations were carried out using BioCro based on 2002 and 2005 environmental data for Champaign, Illinois. Maximum yield is 4.15 Mg ha^{-1} for the control and 3.05 Mg ha^{-1} for the nitrogen-fixing version.

Discussion

Currently, there are only two other models that simulate the entire metabolism of a nodulated legume (Pfau *et al.*, 2018; diCenzo *et al.*, 2020). Both of those models are based on *M. truncatula*, a temperate legume species that has indeterminate nodules. The current work presents the first metabolic model of a nodulated food crop, providing precise predictions on the metabolic requirements of N-fixing *B. diazoefficiens* within a soybean nodule (Fig. 3) while taking into account the rest of plant metabolism (shoot and root). This provides a detailed account of whole-plant metabolism during N-fixing symbiosis for a tropical legume species with determinate nodules. We extended upon our FBA predictions of a soybean plant by implementing the predictions into a multiscale semi-mechanistic crop model to estimate the effect of N fixation on crop yield with and without soil nitrogen supply.

Supporting the accuracy of the FBA model, many of the nodule flux predictions (Fig. 3) were consistent with experimental evidence and analyses. This includes: (1) malate being the primary carbon source used by bacteroids over other C_4-dicarboxylates (Udvardi *et al.*, 1988; Udvardi & Poole, 2013; Mitsch *et al.*, 2018), (2) the transfer of molybdate, sulfate, iron, and homocitrate to bacteroids which are required to form the FeMo cofactor of the nitrogenase complex (Winter & Burris, 1976; Hakoyama *et al.*, 2009; Udvardi & Poole, 2013), and (3) assimilation of phosphate from the soil, consistent with experimental reports of high phosphate content within nodules (Sa & Israel, 1991; Gunawardena *et al.*, 1992; Sulieman & Tran, 2017). The FBA simulations also predicted high levels of oxygen import to the bacteroid and a high flux of water from the bacteroid into the nodule, consistent with high flux through the electron transport chain and oxidative phosphorylation to support the high energy demand of N fixation. FBA results predict that the nodule exports allantoin over allantoate. This is consistent with published literature. Pélissier *et al.* (2004) found that overexpressing the common bean PvUPS1 transporter leads to a preference of allantoin transport over allantoic acid, while Carter & Tegeder (2016) found that when overexpressing PvUPS1 in soybean, the ratio of allantoin : allantoic acid is increased in the xylem. Their study also found that N export from nodules is a rate limiting step in nitrogen symbiosis and that increasing N export enables a higher N fixation rate and higher seed yield per plant. Finally, the FBA simulations predicted that the nodule must provide the bacteroid with additional protons in order to maximize plant growth, as also predicted to occur in the *M.truncatula-S.meliloti* FBA model (diCenzo *et al.*, 2020). This is also consistent with work by Udvardi *et al.* (1991), who identified the presence of a H^+- ATPase that pumps H^+ into the peribacteroid membrane to create a membrane potential (Udvardi & Day, 1989). In future work, measuring key metabolic fluxes in soybean nodules (i.e. C_4 dicarboxylate donation, ammonia secretion, ureide export, and respiration) along with relative growth rate under varied soil nitrogen treatments will provide important information to further validate the accuracy of the nodulated soybean FBA model.

Our model predicts soybean growth rates that fall within the experimentally determined range of 0.05−0.1 g g^{-1} DW d^{-1} (Ghazali & Cox, 1981; Wells, 1993; Purcell *et al.*, 1997; Malek *et al.*, 2012), with lower RGRs being measured as the number of days after sowing increases (Tandale & Ubale, 2007; Arif *et al.*, 2010; Malek *et al.*, 2012). The model predicts that in conditions where the rate of photosynthesis is the growth limiting variable, N-fixation costs a soybean plant slightly less than a third of its RGR compared with growth in which all N is provided as ammonium from the soil. This is similar to the decrease in RGR predicted using a FBA model of nodulated *M. truncatula* (diCenzo *et al.*, 2020). Incorporating the FBA-determined cost of N fixation into Soybean-BioCro reduced yield by *c.* 27% which is not unreasonable based on the meta-analysis of Ciampitti & Salvagiotti (2018). However, N-fixation was predicted to allow soybean to grow and yield on a soil with no available N, and to achieve a higher yield than a non-nodulating plant at all soil N supply rates up to 8.97 μmol NH_4 g^{-1} DW h^{-1} (Fig. 4).

The model provides an estimate for the costs of N fixation, although the true cost could vary with host plant genotype and symbiotic partnership. It is possible that the yield costs predicted by FBA and Soybean-BioCro are underestimates as the FBA model assumes metabolism follows the optimum route to maximize growth. If less optimal routes are followed in reality, then higher costs would result. The model also assumes that the bacteroid uses its plant supplied carbohydrate to maximum efficiency in returning N to the plant. However, cheating, where some bacterial strains in nodules use the carbohydrate but deliver smaller amounts or no N, does occur (Sachs *et al.*, 2010; Jones *et al.*, 2015; Regus *et al.*, 2017).

In the other direction, there are many reasons for why the predicted yield costs might be overestimates. The FBA model

only provides an estimate for a distinct point in time. It has been shown that N fixation reduces in the later stages of a plants life, meaning that the metabolic cost of the symbiosis might not always remain as high as in the FBA simulations (Ryle *et al.*, 1979; Twary & Heichel, 1991). In addition, the 30% decrease in RGR was calculated based on simulations in which the plant obtains zero nitrogen from the soil. The costs of symbiotic N fixation would be lower in fields where soil N levels are nonzero, for example due to fertilization of a previous crop, the actions of free-living microbes, and mineralization of N-containing organic matter. Moreover, our comparison was against a plant obtaining all of its N as ammonium from the soil. This was to allow a direct comparison as ammonium is the result of N reduction in the nodule. Second, the dominant forms of N fertilizer in agriculture are anhydrous ammonia, ammonium salts, or urea, which hydrolyses in the soil to ammonium and bicarbonate (FAOSTAT, 2023). However, we recognize that a significant portion of this fertilizer will be microbially oxidized to nitrate, which is readily assimilated by plants (Beeckman *et al.*, 2018) but which must be converted back to ammonium by the plant at a cost of 3 NADPH.H equivalents per nitrate. In a previous model of the metabolism of *M. truncatula* (diCenzo *et al.*, 2020), the RGR cost of N fixation is reduced from 29% to 24% when the reference is changed from a plant assimilating all of its N from ammonium to one solely using nitrate. However, the cost of nitrate assimilation is also influenced by environmental factors. A large amount of nitrate reduction occurs in the leaf, using NADPH.H equivalents and ATP produced by chloroplast electron transport (Liu *et al.*, 2022). In high light, photosynthetic carbon assimilation will be saturated, and therefore, there would be no cost in using this excess reductive power for converting nitrate to ammonium. In the future, it would be interesting to further develop our model to predict the impact of varying the N source on soybean growth rates and yield.

Another consideration is that Soybean-BioCro is parameterized based on Soybean in Illinois, USA, which is among the most productive locations world-wide (Hartman *et al.*, 2011). Our analysis assumes that crop production is limited by photosynthesis, that is production is source-limited and so any photosynthate diverted to N fixation will lower overall production. Clearly, this is not always the case, and a crop could instead be sink limited, that is there is insufficient capacity to utilize the photosynthate formed in plant growth and yield. In an extreme case of sink limitation, N fixation would simply be utilizing photosynthate that growth cannot. Are our major crops sink limited today? Artificial elevation of CO_2 around a crop boosts photosynthesis by accelerating carboxylation by ribulose- 1 : 5-bisphosphate carboxylase/oxygenase and by competitively inhibiting the oxygenation reaction (Long *et al.*, 2004). This artificial elevation of CO_2 provides a test of sink limitation. Season-long elevation of CO_2 results in large increases in yields of most crops and particularly large increases in the most recently released cultivars, suggesting that breeding has removed much sink limitation (Kimball, 1983; Ainsworth & Long, 2005, 2021).

Some studies have suggested that nodulated plants have higher rates of photosynthesis compared with non-nodulated plants (Bethlenfalvay *et al.*, 1978; Brown & Bethlenfalvay, 1987, 1988). This work was with older cultivars that may well have been sink limited and so the presence of the additional sink provided by N fixation could have prevented end-product inhibition of photosynthesis. Increasing CO_2 uptake within our FBA model increased N fixation and relative growth rates, allowing the plant to reach a growth rate equivalent to that of fertilized soybean. This is consistent with many studies that show that increases in photosynthesis increase nodule biomass and N fixation (Rogers *et al.*, 2009; Lam *et al.*, 2012; Sanz-śaez *et al.*, 2015; Li *et al.*, 2017; Friel & Friesen, 2019) and demonstrates the potential for the costs of symbiotic N fixation to be offset by increases in the rate of photosynthesis. Likewise, the costs of N fixation could be offset if the plant were to redirect a portion of the photosynthate used in root exudates to its nodules. Finally, rhizobia can have plant growth-promoting impacts independent of nitrogen fixation that could at least partially compensate for the metabolic costs of the symbiosis, where growth is not limited by the supply of photosynthate (Noel *et al.*, 1996).

Despite assuming there is zero N available within the soil, the predicted yield of a N-fixing soybean was still 47% higher than that in nutrient scarce regions of Africa where average yields range from 2.066 Mg ha^{-1} without any inputs to 2.39 Mg ha^{-1} with inoculation (see Dataset S3). Based on VCR predictions, results show that inoculation is more profitable than fertilizer for African farmers despite the lower yield. The profitability increases further when considering that yearly application of inoculant may not be required. Moreover, inoculation is likely to be particularly beneficial when there is limited access to additional N inputs or other environmental conditions preclude realizing the full yield benefit of N fertilizer, which is quite often the case (Khaitov & Abdiev, 2018; Moretti *et al.*, 2018). These computational results are consistent with the multiple experimental studies that have examined the benefits of inoculating soybean with rhizobia in Sub-Saharan Africa (Abaidoo *et al.*, 2015; Ulzen *et al.*, 2016; Engoke & Boahen, 2018; Ulzen, 2019; Awuni *et al.*, 2020).

The carbon cost of N fixation – defined here as g C lost from nodules through CO_2 per g N fixed by nitrogenase – for the soybean FBA model is 4.13 g C g^{-1} N and ranged from 4.13 to 4.15 g C g^{-1} N when varying the root : shoot ratio. These predictions fall within the range of values observed through experimental measurements for soybean (Warembourg, 1983) along with theoretical estimations based on ATP requirements (Minchin & Witty, 2005). Furthermore, the carbon cost predicted here for soybean, a ureide exporter, is slightly lower than the 4.2 g C g^{-1} N predicted using a FBA model of *M. truncatula*, an amide exporter (diCenzo *et al.*, 2020). However, this difference could not be explained by the form of the N export product as FBA and FVA results showed that the carbon cost of N fixation decreased and RGR increased when soybean was required to export amides in place of ureides. This appeared to be due directly to changes in

carbon use efficiency in the amide vs ureide synthesis pathways. Synthesis of asparagine involves PEP carboxylation, resulting in a quarter of the carbon in asparagine being derived from dark CO_2 fixation, thus reducing net nodule CO_2 production. By contrast, the conversion of uric acid to allantoin generates a CO_2 molecule, increasing the net CO_2 loss by the nodule. Overall, our simulations highlight that despite ureide synthesis being more ATP efficient, ureides as a N export product are less efficient in terms of carbon use (Table 1; Fig. 5). Consequently, at least in some conditions, the use of ureides as a N export product may increase costs and decrease plant RGR. Interestingly, Le Roux *et al.* (2009) showed that under phosphate stress, there was an increase in soybean nodule respiration and the ratio of ureides to amino acids.

There is significant interest in engineering N-fixing symbiosis into cereals (Kasting & Siefert, 2001; Rogers & Oldroyd, 2014). Our results suggest that the effect of N fixation on yield could be much smaller in a cereal, using the example of maize. Using our soybean model, but assuming the N content of maize, indicated that a plant obtaining all of its N from biological fixation would yield *c.* 14% less biomass than one that obtains all of its N in the form of ammonium from the soil. This low cost is likely similar for other cereals, with rice, sorghum, and millet all having very similar N contents to maize (Li *et al.*, 2013). A caveat to this conclusion is that while soybean and maize share many aspects of primary metabolism, they differ in photosynthetic type and in aspects of secondary metabolism. Determining costs more precisely would require developing a metabolic model of nodulated maize. Such a model would be a powerful tool to support the design of a synthetic nodule by guiding the selection of which metabolic pathways to include, such as whether the nodule should use uriedes or amides as the N export product.

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Competing interests

None declared.

Author contributions

SPL, BLH and GCD conceived the study. BLH and GCD built the metabolic model and carried out the plant level simulations. PB and LJS gathered data used to build the metabolic model via experiments. MLM ran the crop level simulations. BLH, SPL and GCD interpreted the results. BLH, SPL, GCD and MLM wrote the manuscript. All authors revised the manuscript and approved the final version.

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Data availability

The full code for model reconstruction can be found on GitHub (https://github.com/bop15bh/SoybeanFBA), while the datasets used are provided in Datasets S1, S2, and S3.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 List of the model reactions, metabolites, and added amide model reactions with their associated formulas, database IDs, gene rules, and subsystems.

Dataset S2 Biomass composition data and calculations required for root, shoot, and nodule biomass reactions.

Dataset S3 Data provided by Soybean Innovation Lab for average yield for soybean in several African countries with and without inoculant.

Methods S1 Supplemental methods describing the model construction and additional analyses.

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