Metabolic modeling reveals synergistic growth benefits of engineering maize symbiosis with rhizobia and arbuscular mycorrhizal fungi

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Key words: Flux Balance Analysis, Arbuscular Mycorrhizal Fungi, N2 fixation

Abstract

Engineering N_2-fixing rhizobia symbiosis in cereals and enhancing association with arbuscular mycorrhizal fungi (AMF) are two strategies being pursued to improve agricultural sustainability. However, the metabolic fluxes and consequent costs and benefits of these strategies in a cereal crop have not been examined. We developed a metabolic model describing how the relative growth rate of *Zea mays* is impacted by hypothetical association with the rhizobium *Bradyrhizobium diazoefficiens* and the AMF *Rhizophagus irregularis*. We found that the lower N content of cereal crops makes the relative growth rate cost associated with acquiring nitrogen from N_2-fixing rhizobia smaller than in legumes. Our model also predicts positive synergies between rhizobia and AMF under nutrient-limited conditions but negative synergies under nutrient, particularly phosphorus, replete conditions. These findings indicate that these bioengineering strategies could improve cereal crop yields and may achieve greater gains in tandem, but soil nutrient status of target sites should be considered.

Significance Statement

Fertilizer application is responsible for substantial environmental impacts and represents a significant cost to farmers, both barriers to sustainable agricultural development. Engineering cereal crops to associate with N_2-fixing rhizobia and enhancing existing associations between crops and arbuscular mycorrhizal fungi (AMF) are two strategies that could address these challenges. However, quantitative predictions of their potential effectiveness have not been determined. We use metabolic modeling to demonstrate that engineering maize to associate with N_2-fixing rhizobia could be a highly metabolic and cost-effective strategy for sustainably supplying nitrogen to cereal crops. We also develop and experimentally validate a model of plant-AMF carbon and nutrient exchanges and demonstrate that AMF and rhizobia symbioses can act synergistically under nitrogen- and phosphorus-deprivation to improve crop growth.

Introduction

Agricultural production poses a huge challenge to global efforts to address the intertwined problems of anthropogenic climate change and sustainable agricultural development. Approximately 19% of greenhouse gas emissions can be attributed to agriculture and deforestation (1). CO_2 emissions associated with nitrogen fertilizer production and the release of N_2O from agricultural fields post-application contribute heavily to these emissions (2–4). Additionally, these fertilizers represent a substantial portion of the cost of agriculture and recent volatility in fertilizer prices has exacerbated food insecurity in Africa (5). Increases in the cost of urea in Africa specifically have been linked with increased deforestation due to expanded agricultural land use (6).

Motivated by our growing understanding of the role of beneficial soil microbes in agriculture (7), two promising approaches have been proposed to address these challenges. The first is to engineer associations with N_2-fixing bacteria into crops that do not currently have this capacity, a research effort that has been active for many years (4, 8, 9). The second is to enhance the existing capacity of crops to associate with and take up nutrients from arbuscular mycorrhizal fungi (AMF)(10). The former strategy would allow non-leguminous plants, in symbiosis with N_2-fixing rhizobia, to fix nitrogen instead of relying on external nitrogen application. The latter

strategy would increase crop access to soil nitrogen and phosphorus through symbiotic exchange with AMF, reducing the amount of fertilizer necessary for optimal crop growth. Both strategies could reduce the climate impacts of agriculture and facilitate sustainable agricultural development in food-insecure regions.

The addition of rhizobia sustainably improving crop yields could be most effective in a cereal crop with low nitrogen content, like maize (*Zea mays*). Due to this lower nitrogen requirement, a previous analysis predicted that, compared with a legume like *Glycine max*, maize engineered to host rhizobia might suffer from significantly lower reductions in relative growth rate (RGR) when in symbiosis with rhizobia (11). However, this reduced RGR penalty was inferred through a simple comparison of the nitrogen content of maize and soybean, rather than through a full metabolic modeling analysis. This analysis also did not account for changes in tissue carbon and nitrogen content during maize development, which may result in the growth penalty varying over time.

The potential benefits of engineering cereal crop associations with rhizobia or AMF raises the question of how effective a stacked strategy incorporating both rhizobia and AMF could be for boosting sustainable crop yields. The potential growth improvements from N_2-fixation by rhizobia in cereal crops may be limited in some soils by low phosphorus availability. AMF improve plant phosphorus uptake (12) and could allow plants to fully realize rhizobia-mediated growth benefits in phosphorus poor soils. A meta-analysis on the effects of AMF-inoculation on plant growth found that in laboratory studies of AMF fungi, N_2-fixing forbs benefited more from AMF-inoculation than C4 grasses did, suggesting possible synergy between these symbiotic associations (13). However, a detailed analysis of the predicted benefits of engineering cereal crop associations with AMF and rhizobia in isolation and how they interact has not yet been conducted.

In this study, we use genome-scale models of the metabolic networks of the C4 grass *Zea mays*, the N_2-fixing rhizobium *Bradyrhizobium diazoefficiens,* and the AMF *Rhizophagus irregularis*, to characterize the costs and benefits of rhizobia and AMF on crop growth in isolation and together. First, we develop a model of maize central metabolism that accurately reproduces essential growth characteristics of the plant. We calculate the carbon cost of biological nitrogen fixation in this system, confirming previous predictions that the cost should

be lower than in soybean, but find that this cost varies across plant development. Next, we develop and experimentally validate an integrated model representing the exchange of nutrients between maize and *R. irregularis*. Combining the models describing the AMF and rhizobia associations, we predict strong synergy between these strategies under low phosphorus and nitrogen conditions in terms of their positive impact on plant RGR (see **Figure S1** for a graphical representation of the model). Under phosphorus-replete soil conditions, however, we predict antagonism between these approaches.

Results

Construction and validation of a multi-tissue model of maize central metabolism

We constructed a multi-tissue metabolic model of maize central metabolism using a bottom-up reconstruction approach using the central metabolism of *Arabidopsis thaliana* as a base(14). The model features a biomass equation representative of maize(15), separation of the mesophyll and bundle-sheath cells to simulate C4 photosynthetic fluxes, and separate root and shoot tissue compartments connected by inter-tissue metabolite exchanges based on a previous study(16). Where necessary, additional biochemical reactions were added to the model to allow the synthesis of all biomass components **(Dataset S1)**.

We compared key outputs of our model to literature estimates and found them highly consistent **(Table 1)**(17–20). Taken together, these validation results indicate that the model captures key photosynthetic details and the overall energetic efficiency of maize central metabolism. Local sensitivity analysis was performed on this base plant model and the other models presented in this study and shows that outputs of interest are robust to small variations in key parameter values (**Figure S2)**.

The RGR penalty incurred by N_2-fixation in maize is lower than in non-cereal crops and decreases across development

A model of *Bradyrhizobium diazoefficiens* (21) was connected to the maize model, with a new nodule tissue compartment, to investigate the potential benefits and drawbacks of creating an association between maize and an N_2-fixing rhizobium. By comparing simulation results with and without the bacteroid and nodule tissues present, we quantified the RGR penalties of using

N_2-fixation in lieu of soil nitrogen uptake across a range of soil nitrogen availabilities and across several different developmental stages in maize **(Table 2; Figure 1)**.

The RGR penalty associated with maize acquiring all of its nitrogen from N_2-fixation versus soil uptake ranges from ~12.8% in the seedling stage down to 3.4% at the silking stage, which is qualitatively consistent with prior estimates (11). The predicted carbon cost of N_2-fixation ranges from 2.6 g C g N^{-1} at the seedling stage to 3.1 g C g N^{-1} at the silking stage. This decreased efficiency results from decreased use of N_2-fixation during the silking stage when nitrogen requirements begin to decrease (**Table 2**) and fixed non-growth associated maintenance in the nodule and bacteroid tissues. These values are consistent with the range of empirically measured g C g N^{-1} values in the literature for soybean (2.5 to 7 g C g N^{-1})(22).

Value Cost Ratio comparisons indicate that a nitrogen-fixing maize crop could be profitable in both the United States and sub-Saharan Africa

To assess the economic viability of inoculating a hypothetical nodulating maize plant with the modeled RGR penalties, we calculated the Value Cost Ratio (VCR) of fertilizing maize with typical nitrogen fertilizer and the VCR of adding rhizobia inoculum with yield and price estimates for the United States (USA) and sub-Saharan Africa (SSA) **(Table 3)**.

Our results show that in both the USA and SSA, the potential profitability of a nitrogen-fixing maize crop is greater than that typically observed with regular nitrogen fertilizer additions, with a VCR of 45.4 vs 3.4 for the USA and a VCR of 8.1 vs 1.9 for SSA respectively. The greater potential profitability in the USA is driven by the much lower price of inoculum in the USA (\$9.9 ha^{-1})(23) as compared with SSA (\$38 ha^{-1})(11, 24). This large difference in inoculum cost contrasts with the similar nitrogen fertilizer costs in the USA (\$198 ha^{-1})(25) and SSA (\$205 ha^{-1})(26).

Experimentally validated plant-AMF model relates biomass improvements in maize to nutrient and metabolite exchanges

To understand the relationship between the nutrient uptake benefits mediated by AMF and biomass benefits observed in plants, we built a multi-species plant-AMF model. In this plant-AMF model, the plant exchanges carbon in the form of palmitate and glucose with the AMF in exchange for nitrogen and phosphorus taken up by the AMF. Key parameters for this model

include the percent improvement of external nitrogen and phosphorus that the plant-AMF system can achieve compared to the base plant uptake, and the amount of fungal biomass necessary to achieve these uptake rate improvements.

The growth benefits realized by maize from AMF-mediated nutrient uptake are maximized in nitrogen-replete and phosphorus-depleted conditions (**Figure 2A)**. Accordingly, because our model allocates carbon investment to the fungus in such a way to maximize plant RGR, carbon investment is maximized under those same conditions. This carbon investment decreases, eventually to zero, as nitrogen and phosphorus availability increases and plant uptake alone becomes sufficient to achieve maximal plant RGR **(Figure 2B).** These predictions are consistent with the trade-balance model of the AMF symbiosis, which describes differing levels of growth benefit conferred by AMF on plants in terms of nutrient availability, relative nutrient uptake capacities of plants and AMF, and the biomass composition of plant and fungal tissues (12). This model describes the plant-AMF system using economic trade principles, making the exchange rate of carbon and nutrients crucial. By comparing the carbon allocated by the plant to the fungus and the nutrients received in return in our model, we can estimate the stoichiometry of carbon and nutrient exchange between the plant and AMF partner is 28 g C g P^{-1} and 6.3 g C g N^{-1} (**Dataset S2).**

Our local sensitivity analysis **(Figure S2B-C)** shows that the stoichiometry predictions from our model vary linearly with the assumed amount of AMF dry weight needed per unit plant dry weight. This means that the accuracy of our estimate of the AMF dry weight needed will determine how reasonable the g C g N^{-1} and g C g P^{-1} estimates are. Using data on the hyphal length density of AMF under conditions where phosphorus uptake benefits are observed in plants (27) along with literature estimates on the density and fresh-to-dry weight ratio of fungal tissue (28), we estimated that AMF biomass amounting to 9% of the total plant biomass is necessary to support maximum nutrient uptake benefits. This is slightly higher but qualitatively similar to a previous study's estimate of ~6% (29). With this assumption, the range of carbon investment predicted by the model, 0 to 5% of plant carbon (**Figure 2B**), is consistent with values reported in the literature (2.3 to 10% for *R. irregularis* (30)).

To assess whether the growth-rate improvements predicted by our model are consistent with those observed in the field, we used empirical time-series measurements taken from field-

grown maize hybrids with and without a mutation in the gene *Castor* that results in an inability to associate with AMF (31). These plants were grown in phosphorus-replete (100%+ of necessary P) and nitrogen-limiting (~84% of necessary N) soil conditions (**Dataset S3**). The observed RGR benefit when comparing AMF- and AMF+ plants in this experimental dataset is 30.4%, which compares well with the predicted value of 19%. We also assessed the difference in final shoot dry weights between the AMF- and AMF+ plants and found that the ability to interact with AMF conferred a 22.6% benefit. Both measures show that our model accurately predicts AMF-mediated growth benefits under phosphorus-replete, nitrogen-limited conditions. Under phosphorus-limited conditions our model predicted a 108% improvement in RGR **(Figure 2A)** which compares with the 130% improvement in shoot dry weight in AMF+ vs. AMF- maize plants measured in a previous study (27). That study did not report time-series dry weight data, preventing us from making a direct comparison with estimates of RGR.

Rhizobia and AMF provide synergistic benefits under nutrient-poor conditions, but are antagonistic under nutrient-replete conditions

To characterize interactions between the use of rhizobia and AMF, we constructed a model featuring maize in association with both symbionts. We used this model to predict growth benefits across different phosphorus and nitrogen levels and compared them with the additive benefits of using each strategy in isolation. By calculating the difference between the RGR benefits predicted by the combined model and the additive predicted benefits from the individual plant-symbiote models (**Figure S3**), we were able to quantify the synergy and antagonism between these strategies as a function of nutrient availability.

Under phosphorus depletion and low to medium nitrogen availability our model predicts that the two symbiotic relationships work synergistically by relaxing the nutrient limitation imposed by both phosphorus and nitrogen and allowing the plant to achieve near-optimal RGR with less than 100% N and P availability. Maximal RGR improvement is observed under conditions of phosphorus depletion with low to moderate nitrogen availability (**Figure 3B**). In this new maximal region, the relative benefit increased from 23% using only AMF or 400% using only rhizobia to 789%, corresponding with an absolute increase of RGR of 0.076 day^{-1}. The synergism between the symbiotic relationships is largest (409%) when nitrogen availability is at 10% and phosphorus levels are at 40% (**Figure 3C**). The predicted RGR of plants grown

under these nutrient conditions with both the AMF and rhizobia symbiosis is 0.080 day^{-1}, roughly double the value predicted assuming the rhizobia and AMF are non-synergistic (0.041 day^{-1}). Comparing this with the RGR predicted for plants without either symbiont but with 100% of their nutrient needs met (0.091 day^{-1}) suggests that plants could achieve ~83% of their maximum RGR with only 10% and 40% of optimal N and P availability. Under these conditions, plants get the majority of their N from their rhizobial symbionts (~85%) and only (~3%) from their AMF symbionts **(Dataset S2)**, consistent with the lower g C g N^{-1} associated with rhizobia versus AMF.

Maximum antagonism (-21%) is observed under phosphorus-replete conditions, whereas the model predicts negligible antagonism (less than -1%) under nitrogen-replete conditions. The magnitude of the positive synergy under moderate- to low-nitrogen availability and moderate phosphorus availability, expressed in terms of relative change, (48-409%) far outweighs the worst antagonisms seen (0-21%). However, the worst antagonisms are observed under phosphorus-replete conditions, some of which support high basal RGR. Because of this, the worst antagonisms in terms of absolute RGR differences under phosphorus-replete conditions (0 to -0.016 day^{-1}) are comparable to the highest positive synergy values (0.021 – 0.039 day^{-1}).

Discussion

Both the addition of rhizobia to cereal crops and the enhancement of AMF-associations and accompanying nutrient-uptake benefits are strategies being pursued to sustainably improve agricultural production (4, 10, 32), so the question of whether these strategies complement or hinder each other is crucial. Our results predict that synergy between these strategies roughly doubles the relative growth rate of maize plants under severe N deprivation and moderate P availability compared to a scenario where no synergy between these strategies exists. We also predict significant antagonism between these strategies under phosphorus-replete conditions. These findings suggest that soil nutrient status should be considered when developing and deploying crops with added rhizobia and enhanced AMF associations.

In addition to our findings on synergy, we explored the efficiency of engineering rhizobia N_2-fixation and enhanced AMF association individually. We predict a lower RGR penalty (3.6 – 12.8%) than previous work (~14%) that predicted the penalty in maize by comparing the overall (grain + stover) N content of soybean (3.1%) and maize (1.4%) at harvest (11, 33, 34). We also

find that the RGR penalty is highest earlier in vegetative growth when the nitrogen content of plant tissue is higher, so focusing on end-point measurements of plant biomass composition may not give the best estimate of the cost of nitrogen fixation over a growing season. However, our results support the idea that cereal crops should experience smaller RGR losses than legumes when relying on N_2-fixation **(Table 2),** making this an attractive bioengineering approach to sustainably improve cereal crop yields.

From a technology adoption perspective, our VCR analysis (**Table 3**) indicates that inoculation of a nitrogen-fixing maize plant could be a profitable alternative to the use of nitrogen fertilizer in both the USA and SSA. However, the profitability would be greater in the USA due to the almost fourfold greater price of inoculum on a per hectare basis in SSA as compared with the USA (11, 26). This suggests that, if nitrogen-fixing maize is developed, reducing the cost of inoculum for cultivators will be important for driving the adoption of this technology in SSA.

On the AMF engineering side, this study presents the first metabolic model describing the exchange of carbon, nitrogen, and phosphorus between a plant and AMF. Our plant-AMF framework reproduced empirically observed RGR benefits and predicted carbon investments consistent with prior literature (30). This framework represents a promising platform for computational investigations into plant-AMF symbiosis. This framework could be further strengthened with additional information on below-ground AMF biomass quantities and the interdependence of nitrogen uptake, phosphorus uptake, and plant biomass gains. The data currently available is limited and has widely varying estimates of the impacts of AMF, particularly regarding AMF-mediated nitrogen uptake (35–37).

The models presented in this study could be expanded upon to further investigate the costs and benefits of symbiosis on maize. The *R. irregularis* model could be expanded to account for the different tissue types seen in that organism (38). Further, in our framework, the fungus is treated as carbon-limited and does not compete for external nutrients with the plant, so the parasitism predicted by the trade-balance model (12) under nutrient-replete conditions cannot be observed. Explicitly modeling this resource competition could address this. To quantify the impact of these symbioses on grain production, a developing kernel tissue compartment could be added to the maize model. Finally, incorporating dynamic changes to RGR corresponding to the

262 different developmental stages with a crop growth model would allow us to translate RGR
263 changes into yield predictions.

264 This work presents the first analysis of the predicted metabolic flux costs and benefits of
265 engineering N_2-fixation in a cereal crop by rhizobia alone and together with AMF. Our results
266 support previous predictions that cereals represent an attractive target for engineering to
267 associate with rhizobia due to their lower nitrogen content. We also developed the first
268 experimentally validated metabolic model describing the exchange of carbon, nitrogen, and
269 phosphorus between plants and AMF. Creating a combined plant-AMF-rhizobia model, we
270 predicted substantial synergy between the two engineering strategies under moderate
271 phosphorus-depleted and highly nitrogen-depleted soil conditions, though this is counterbalanced
272 by antagonism between these strategies under phosphorus-replete conditions. Our results indicate
273 that these engineering strategies could efficiently and economically improve cereal crop yields.

274

275 **Materials and Methods**

276 *Maize shoot/root model construction*

277 A bottom-up reconstruction of *Arabidopsis thaliana's* metabolism (14) was used as a base
278 representation of plant central metabolic processes. As done in a previous C4 metabolic
279 modeling study (39), this model was duplicated to create separate bundle sheath (BS) and
280 mesophyll (M) models. Other additions and refinements of the bottom-up reconstruction (14)
281 described in the C4 modeling study (39) were also made to improve the accuracy of the model,
282 with the exception of the latter's modifications to the chloroplast ATP synthase reaction, since
283 the original model already correctly captured the c-subunit stoichiometry and consequent
284 ATP/proton stoichiometry typical of plant systems (40).

285 These models were then connected by transport reactions representing exchanges
286 between BS and M cells through the plasmodesmata and flux through cytosolic
287 phosphoenolpyruvate carboxylase and mitochondrial malate dehydrogenase 1 were disallowed,
288 based on the methods described in Blätke & Bräutigam 2019 (39) to ensure that the flux map of
289 the resulting model lines up with canonical carbon assimilatory fluxes through the NADP-ME

290 subtype characteristic of maize (41). As in Holland et al. 2023 (11), based on earlier literature
291 derivations (42), an upper bound of 2920 μmol g^{-1} DW h^{-1} on photon uptake was imposed.

292 Another duplicate of the central metabolic model (14) was then made to represent the
293 plant's root tissue. Metabolite exchanges were adopted from Chowdhury et al. 2023 (16). All
294 metabolite exchanges between the shoot tissue model and the environment, apart from CO_2, O_2,
295 and light, were disallowed. The root tissue model was treated the same except with the additional
296 capacity to take up ammonium, orthophosphate, water, and dihydrogen sulfide. The
297 orthophosphate uptake reaction was modified based on the fact that direct phosphate uptake is
298 thought to occur through proton symporters (43). Two protons were assumed to move across the
299 cell membrane for each phosphate (44).

300 Base biomass compositions for the root and shoot tissue were adapted from Saha *et al.*
301 2011 (15), with additional reactions from KEGG (45) added to produce biomass components not
302 covered by the original *Arabidopsis* central metabolism model (14). A list of added reactions can
303 be found in **Dataset S1**. The ratio of shoot to root biomass was constrained to a value of 90:10
304 (46).

305 Growth-associated maintenance costs (GAM) and non-growth associated maintenance
306 costs (NGAM) for maize were calculated from literature data (47) and modeled either as generic
307 ATP or glucose burning at a specified rate **(Dataset S4)**. Growth-associated maintenance was
308 calculated as 19 mmol ATP gDW^{-1} d^{-1}, lower than the assumed 30 ATP gDW^{-1} d^{-1} featured in
309 many other plant metabolic models (48). The NGAM cost was calculated as 5.76 mmol ATP
310 gDW^{-1} d^{-1}. All maintenance costs were distributed across tissues according to their proportions of
311 total dry weight.

312 *Nodulated maize model construction and analysis*

313 The workflow previously used in Holland et al. 2023 (11) was adapted to create a multi-species
314 model with the maize C4 root/shoot model described above as the plant component and the GEM
315 of *Bradyrhizobium diazoefficiens* as the N_2-fixing bacterial symbiont (21).

316 First, a duplicate of the root model, representing the tissue of a hypothetical maize
317 nodule, was made with exchanges between it and the root limited as in Holland et al. 2023 (11)
318 and as listed in **Dataset S5**. Next, transporters describing the exchange of dicarboxylates and

ammonium were added between the nodule model and the *B. diazoefficiens* model to reflect known exchanges from the literature (11). Allantoate is thought to be the primary form of nitrogen exchanged between nodule tissue and the plant root, so reactions describing allantoate biosynthesis and degradation were added to the model from KEGG (45).

The non-growth associated maintenance costs of *B. diazoefficiens* were assumed to be the same as those for the *Medicago truncatula* bacteroid *Sinorhizobium meliloti*: 2.52 mmol gDW^{-1} h^{-1} (49). Assuming that the bacteroid constitutes 2% of the biomass of the overall system (11), this value was scaled to 1.21 mmol gDW^{-1} d^{-1}. At steady-state we assume that the bacteroid is not accumulating biomass, so growth-associated maintenance costs were ignored.

AMF maize model construction and analysis

We used the previously published and validated GEM of the AMF *Rhizophagus irregularis* to represent the fungal partner in a plant-fungus symbiotic relationship (38). To make the *R. irregularis* model dependent on the plant for its carbon and energy needs, all external uptakes were disabled except for those with oxygen, carbon dioxide, dihydrogen sulfide, ammonium, phosphate, and a number of mineral micronutrients necessary for biomass accumulation. Based on literature evidence, only a small number of metabolites were allowed to be exchanged between the fungal and plant root models (43) **(Dataset S5**). From the plant, sugar (in the form of glucose) and palmitic acid were allowed to be transported to the AMF. From the fungus, phosphate and ammonium were allowed to be transported, with a cost of 0.5 ATP per orthophosphate transported (44).

Recalculation of biomass coefficients to account for changing tissue C:N ratios

Published C:N ratio data (50) was used by modifying the base maize biomass composition from Saha et al. 2011 (15) to match the measured shoot and root C:N ratios. The nitrogenous biomass (NB) and non-nitrogenous components of the biomass (B) were grouped together and the appropriate proportion for these groups were calculated as follows:

$$NB_{proportion} = \frac{B_{carbon\ proportion}}{C{:}N * NB_{nitrogen\ proportion} - NB_{carbon\ proportion} - B_{carbon\ proportion}} \quad (E1)$$

Where $B_{carbon\ proportion}$ is the proportion of the weight of the non-nitrogenous biomass that is made up of carbon, $NB_{nitrogen\ proportion}$ and $NB_{carbon\ proportion}$ are the proportions of the weight of the nitrogenous biomass that is made up of nitrogen and carbon respectively, and *C:N* is the desired C:N ratio. We then split the proportion of non-nitrogenous biomass into the individual non-nitrogenous biomass components in a way that maintains their ratios relative to each other from the original biomass equation (15). Finally, these weight proportions were divided by the average molecular weight of each class of compounds to give the coefficients for each in the final biomass equation in units of mmol gDW^{-1} day^{-1}.

$$Nitrogenous\ compounds_{coefficient} = \left(\frac{1000\ mmol}{mol}\right)\left(\frac{NB_{proportion}}{MW_{Nitrogenous\ compounds}}\right) \qquad (E2)$$

$$Coefficient_n = \left(\frac{1000\ mmol}{mol}\right)\left(\frac{\left(1 - NB_{proportion}\right)\left(\frac{Original\ Coefficient_i}{\sum_{i \in Biomass\ Coefficients} Original\ Coefficient_i}\right)}{Average\ MW_i}\right) \qquad (E3)$$

Where $Original\ Coefficient_i$ is the coefficient for a given biomass component *i* in the original biomass equation (15), *Biomass Coefficients* is the set of all of these original coefficients, and $Average\ MW_i$ is the average molecular weight of the constituent metabolites in a group of biomass compounds.

Optimization details

Flux Balance Analysis optimizations were performed, in most simulations, to maximize plant RGR.

$$\max v_{plant\ biomass} \qquad (E4)$$

$$s.t.$$

$$Sv = 0$$

$$LB_j \leq v_j \leq UB_j$$

Where S represents the stoichiometric matrix of the metabolic network model being optimized, *v* is the vector of all fluxes in that network, and LB and UB are vectors of the lower

and upper bound constraints. In this study, all optimizations were followed up by minimization of the sum of fluxes in the network via regularization of the L1-norm (51), often referred to as parsimonious FBA (pFBA) (52), which corresponds to the following optimization:

$$\min \sum_{j \in Reactions} v_j \qquad (E5)$$

$$s.t.$$

$$Sv = 0$$

$$LB_j \leq v_j \leq UB_j$$

$$v_{plant\ biomass} = v_{maximized\ plant\ biomass}$$

Where *Reactions* is the list of all reactions in the network and $v_{\text{maximized plant biomass}}$ is the maximized biomass value obtained from the initial optimization.

Model optimization and analysis

Growth benefits associated with N_2-fixation, AMF association, and both N_2-fixation and AMF association in concert, were estimated by comparison of predicted plant RGR in the base maize model and the appropriate symbiosis models across a range of soil nitrogen and phosphorus uptake rates **(Figure S2)**. All nitrogen uptake by the plant and, in models that feature it, the AMF symbiont is assumed to be in the form of ammonium.

Optimizations of the maize model with an added N_2-fixing bacterial symbiont were performed as in Holland et al. 2023 (11). Briefly, the plant model without the bacterial symbiont is first optimized to maximize RGR and the ammonium uptake necessary to support this maximized growth rate is recorded. This ammonium uptake rate defines the point at which the model's biomass accumulation is no longer limited by ammonium availability. For both the model with and without the associated N_2-fixing symbiont, we then continuously decrease the external ammonium level and evaluate the RGR at each point. We also calculate the "grams of carbon per gram nitrogen," or g C g N^{-1}, which is a measure of the ratio of carbon, in the form of glucose or fatty acids, sent to the fungus from the plant in exchange for nitrogen from the fungus. g C g N^{-1} was calculated as in Holland et al. 2023 (11):

$$C : N\ ratio = \frac{V_c}{2\ V_n} \qquad (E6)$$

Where V_c is the rate of CO_2 release from the nodule tissue and V_n is the flux through the bacteroid nitrogenase reaction.

AMF model optimization and analysis

The AMF model was optimized in a sequential process meant to mathematically encode the following assumptions about the plant/AMF symbiosis:

1. Since the AMF fungus is dependent on carbon from the host plant, the ability of the fungus to confer nutrient uptake benefits to the plant is a function of carbon investment from the plant.
2. To achieve a measured level of nutrient uptake benefits because of AMF inoculation, the modeled system must achieve the same ratio of AMF to plant biomass, with decreasing ratios linearly decreasing the nutrient uptake benefits conferred.

The data for phosphorus uptake benefits in AMF- and AMF+ plants[3], data for nitrogen uptake benefits (53), and hyphal length density (HLD)(27) values were used to estimate AMF biomass necessary to achieve maximal nutrient uptake benefits. We estimated the dry weight of AMF tissue (DW_{AMF}) from the HLD values and assuming the plants were grown in 2,750 grams of soil[3], as described in Equation E7.

$$DW_{AMF} = \left(\pi r^2 \left(HLD_{low\ P}\left(\frac{m\ hyphae}{g\ soil}\right) * 2750\ g\ soil\right)\right) \left(\frac{1.09 * 10^6\ \mu g\ hyphae\ FW}{hyphae\ cm^3}\right)\left(\frac{0.21\ \mu g\ hyphae\ DW}{\mu g\ \ hyphae\ FW}\right)\left(\frac{9.75\ \mu g\ fungal\ DW}{\mu g\ hyphae\ DW}\right) \qquad (E7)$$

Where $HLD_{\text{low P}}$ is the hyphal length density in centimeters reported in the lowest phosphorus condition. The conversion between hyphae fresh weight (FW) and hyphae volume, $\left(\frac{0.21\ \mu g\ hyphae\ FW}{hyphae\ cm^3}\right)$, is taken from literature estimates, as is the fresh weight to dry weight (DW) conversion (28). Finally, given that substantial fungal biomass is contained in non-hyphal tissue like vesicles, we used a reported ratio of hyphal to total (hyphal plus vesicle) biomass (29),

$\left(\frac{9.75\ \mu g\ fungal\ DW}{\mu g\ hyphae\ DW}\right)$, to convert from the hyphal biomass estimate to an estimate of total fungal dry mass.

To optimize the system, the maximal biomass growth of the system without any AMF-mediated nutrient uptake benefits is calculated across a range of phosphate and ammonium uptake rates as described in **Eq. 4 – 5**.

The ratio of the AMF- plants' biomass and the AMF itself in the low-phosphorus condition reported in Sawers *et al.* 2017 (27) was then used to define the following reactions in the model:

$$(Growth_{in})Biomass_{AMF} * AMF_{proportion} \rightarrow P_{uptake} + N_{uptake} \qquad (E8)$$

$$(2.16 * P_n)Orthophosphate_{fungus} + P_{uptake} \rightarrow (2.16 * P_n)Orthophosphate_{plant} \qquad (E9)$$

$$(0.23 * N_i)Ammonium_{fungus} + N_{uptake} \rightarrow (0.23 * N_i)Ammonium_{plant} \qquad (E10)$$

Where $Growth_{in}$ is the maximal biomass accumulation that the plant is capable of in the absence of AMF-mediated nutrient uptake benefits, $AMF_{proportion}$ is the proportion of AMF-to-plant biomass, and P_n and N_i are the non-AMF-mediated plant phosphate and ammonium uptake rates. A single unit of flux through this reaction requires producing the observed amount of fungal biomass relative to plant biomass, yielding a single unit of P_{uptake} and N_{uptake}, which can be used in **Eq. 9** and **Eq. 10** to move 2.16 times the basal phosphate uptake rate or 0.23 times the basal ammonium uptake rate. The upper bound of **Eq. 8** is set to 1, such that no more than 2.16 times the basal phosphate uptake rate and 0.23 times the ammonium uptake rate can be achieved, based off of reported nitrogen and phosphate uptake benefits (27, 53).

With these additions, the full model, plant and AMF partner, is optimized as described in **Eq. 4-5.** In addition to the maximal RGR of the plant, the fluxes of glucose and palmitate from the plant to the fungus and the net CO_2 uptake of the plant are recorded. From these quantities, we calculate the percent of CO_2 from the plant that is invested in the AMF partner:

$$\%\ Carbon\ Allocation = (100)\frac{v_{CO2\ uptake}}{(16\ v_{palmitate}) + (6\ v_{glucose})} \qquad (E11)$$

Where $v_{CO2\ uptake}$ is the plant's CO2 uptake flux and $v_{palmitate}$ and $v_{glucose}$ are the fluxes of palmitate and glucose transport from the plant to the fungus.

AMF growth rate validation

To generate AMF-incompatible (AMF-) maize hybrids for field evaluation, a B73 inbred line homozygous for the *castor-2* mutation (54) was crossed as male to a W22 inbred line also homozygous for *castor-2*. Control AMF-compatible (AMF+) hybrids were generated by crossing the same B73 *castor-2* line as male to an AMF- W22 inbred line carrying a mutation in the maize ortholog of the *pollux* gene (31, 55), with trans-complementation recovering AMF compatibility in the F_1 hybrid progeny. This scheme allowed that both AMF- and AMF+ hybrids were produced from AMF- mothers to reduce any strong maternal effect.

AMF+ and AMF- maize hybrids were evaluated at Russell E. Larson Agricultural Research Center at the Pennsylvania State University (40°42'39.1"N 77°57'11.4"W) within a larger split-split-plot design with 2.5-foot row spacing receiving drip irrigation. Prior to planting, the soil of 90 random rows (14.1 m^2 density) was sampled by pooling soil from the front, middle, and end of rows at a depth of 0-10 cm. Soil samples were then submitted to the Agricultural Analytical Services Lab to determine Total N and Mehlich 3 extractable P by ICP analysis with soil parameters at unsampled rows modeled with kriging interpolation using the 'gstat' R package(56). Seeds were planted on May 18th, 2023, one week after 150 lbs of 19-19-19 (%N-%P-%K) fertilizer was applied. After receiving an additional 300 lbs of urea per acre on June 21st, 2023, AMF+ and AMF- plant biomass was sampled, dried, ground, and analyzed with a dry combustion elemental analyzer to determine Total N content (as described in White et al., 2020)(57) at timepoints T1-T4 (28, 38, 48, and 58 days after emergence) and at harvest on October 4, 2023. To assay AMF colonization in AMF+ (32% colonization; n=117) and AMF- (2% average colonization, n = 57) plants, root tissue was harvested at 8-weeks after emergence to quantify fungal colonization via microscopy following staining. Briefly, ~0.25 g of fine root tissue was collected from 0-0.25 m depth, cleared in 10% KOH at 70°C over 48 hours, rinsed five times in deionized water, incubated in 0.3 M HCl for 15 mins at room temperature, and stained at 96°C for 8 min using a 0.05% w/v Direct Blue 15 Dye staining solution in a 1:1:1 mixture of lactic acid: glycerol: deionized water. Percent root length colonized was then

determined by noting the presence and absence of AMF structures at 40x magnification along 100 intersections on a 1 cm x 1 cm grid (58).

Available N in the field trial was estimated by adding the known N application rate to an estimate of N mineralization from soil organic matter. Soil organic matter (SOM) content was estimated from measured total N, assuming a standard composition of SOM of 5% nitrogen (59, 60). From this, we calculate the contribution of mineralized organic nitrogen as:

$$\left(\frac{2.48e6\ kg\ soil}{ha}\right)\left(\frac{2.2\ kg\ SOM}{100\ kg\ soil}\right)\left(\frac{5kg\ N}{100\ kg\ SOM}\right)\left(\frac{2.0\ kg\ SOM\ mineralized}{100\ kg\ SOM}\right) = 54.56\ \frac{kg\ N}{ha} \qquad (E12)$$

Where the 2.2 kg SOM 100 kg^{-1} soil is derived from the measured total N, the weight of a hectare of soil is estimated assuming a bulk density of 1.24 g/cm^3 in the 0-20cm depth (61), SOM is assumed to be 5% N by weight (59, 60) and 2.0 kg of SOM is assumed to be mineralized per 100 kg of SOM per growing season (62). The 0-20cm depth is used for the mineralization calculation because the majority (~80%) of the root surface area of a maize plant is present between soil depths of 0-20cm (63).

The RGR of plants from the field trial dataset was estimated using the following equation:

$$RGR = \frac{\ln(DW_1) - \ln(DW_2)}{t_2 - t_1} \qquad (E13)$$

Where DW_1 and DW_2 are the shoot dry weight measurements (g) at growth timepoints 1 (t_1) and timepoint 2 (t_2), which are 28 and 38 days after emergence, respectively. These two early growth timepoints were used to calculate RGR to make for as direct a comparison between the AMF- and AMF+ samples as possible by minimizing any potential impact of differing developmental trajectories between AMF+ and AMF – plants.

The average soil phosphorus level across all planted rows was approximately 59 mg kg^{-1} and the average total nitrogen content was 0.11% (kg N kg^{-1} soil) **(Dataset S3).** A previous study (27) showed that both shoot dry weight gains and AMF-mediated growth benefits were previously shown to disappear somewhere between phosphorus concentrations of 15.5 mg kg^{-1} and 53.2 mg kg^{-1}. This indicates that the plants in this study had adequate phosphorus available to achieve optimal or near-optimal growth. Corroborating this idea, one study found that high-

performing maize lines take up an average of 114 kg P_2O_5, or 50 kg P, during the growing season (64), as compared to the 66 kg P that were available to a depth of 20 cm in the samples with the lowest soil P concentration in this study (26.5 mg/kg). This means that the plants assessed in this study received 100%+ of the P necessary to achieve optimal growth. The average total nitrogen content in our plots of 0.11% was measured prior to an application of 187 kg N ha^{-1}. Together, the mineralization of the soil organic matter and the applied N fertilizer amount to the equivalent of an input of 241 kg N ha^{-1}. Assuming that our maize hybrids can achieve similar optimal growth rates as those reported in a previous study assessing optimal N application rates and nutrient uptake rates (64), high-performing maize hybrids will take up an average of 286 kg N ha^{-1}. This means that the plants assessed in this study received ~84% of the N necessary to achieve optimal growth. Together with the P availability, this makes the nitrogen-limiting upper-right-hand quadrant the appropriate comparison between the RGR improvements in **Figure 2A** and our empirical measurements.

Sensitivity analysis

Local sensitivity analysis was performed by varying the values of key input parameters for all models presented in this study by ±10% and then calculating the percent change in (i) RGR, (ii) g C g N^{-1}, and (iii) g C g P^{-1}. The reported sensitivity values are the averages of the percent changes resulting from increasing and decreasing a given parameter value.

Software

Optimizations and model analysis were performed in MATLAB R2020a using the COBRA Toolbox 3.0 (65), the Enterprise IBM ILOG CPLEX solver version 12.10, and the Gurobi Optimizer 11.02.

Acknowledgments

This work was supported by the project Enabling Nutrient Symbioses in Agriculture (ENSA), that is funded by Bill & Melinda Gates Agricultural Innovations (INV-57461) (JAMK, RJ, PS, RJHS, and MLM). PS and RJHS were supported by the United States Department of Agriculture (USDA) project *The genetic basis of maize response to arbuscular mycorrhizal fungi* (2022-67013-38264). RJHS is supported by the USDA National Institute of Food and Agriculture and

Hatch Appropriations under Project #PEN04734 and Accession #1021929. Figures made using BioRender.com.

Data availability

All data, models, and code needed to reproduce the analysis in this study can be found in the online supplemental material accompanying the manuscript and at https://github.com/Matthews-Research-Group/Maize-Rhizobia-AMF_FBA.

Competing interests

None to declare.

Author contributions

JAMK and MLM conceived the study. JAMK built, made predictions with, and analyzed the results of the metabolic models presented in this study. RJ performed the sensitivity analysis. PS and RJHS generated and evaluated AMF compatible and incompatible maize hybrids**.** JAMK, RJ, and MLM interpreted the results. JAMK wrote the manuscript. All authors revised the manuscript and approved the final version.

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Table 1: Photosynthetic and physiological parameter predictions from the model of maize along with qualitative and quantitative data and corresponding references.

Metric	Modeled	Measured	Reference
Organic Carbon Transport from Mesophyll to Bundle Sheath	Malate	Malate	(18)
Carbon Skeleton Transport from Bundle Sheath to Mesophyll	Pyruvate	Pyruvate	(18)
Decarboxylated Species in Bundle Sheath	Malate	Malate	(18)
Relative Growth Rate (g gDW^{-1} d^{-1})	0.082	0.04 – 0.08	(20)
Quantum Yield of Photosynthesis (mol CO_2 mol $photons^{-1}$)	0.0541	0.0534	(17)
Percent of Bundle Sheath PSI Electrons Supplied by Cyclic Electron Flow (%)	65%	84%	(19)

705 **Table 2:** Physiological and metabolic predictions from the *Z. mays* model associated with the N_2
706 fixing rhizobium *B. diazoefficiens*.

Metric	Timepoint / Percent N of Biomass (%)		
	Seedling / 2.70	**Jointing / 2.34**	**Silking / 0.80**
Relative Growth Rate (g gDW^{-1} d^{-1})	0.084	0.084	0.088
Relative Growth Rate Penalty (%)	12.8	11.0	3.4
N_2-Fixation (mmol gDW^{-1} d^{-1})	0.081	0.070	0.025
Nodule CO_2 Efflux (mmol gDW^{-1} d^{-1})	0.50	0.44	0.18
Carbon cost of N_2-Fixation (g C g^{-1} N)	2.6	2.7	3.1

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Table 3: VCR and input parameter values comparing the profitability of fertilizer and rhizobia inoculum application versus using no fertilizer or inoculum amendments in the USA and SSA.

Source	Parameter	Value	Condition	VCR
USA	Fertilizer price **(USD ha^{-1})**	198 (25)	+ Fertilizer	3.4
	Yield with fertilizer **(tonne ha^{-1})**	11.5 (66)		
	Inoculum price **(USD ha^{-1})**	9.9 (23)	+ Inoculum	47.0
	Yield with inoculum **(tonne ha^{-1})**	10.0 (66)[a]		
	Corn price **(USD tonne^{-1})**	141 (67)	Control	
	Control Yield **(tonne ha^{-1})**	6.7 (68)[b]		
SSA	Fertilizer price **(USD ha^{-1})**	205 (26)[c]	+ Fertilizer	1.9
	Yield with fertilizer **(tonne ha^{-1})**	3.0 (26)		
	Inoculum price **(USD ha^{-1})**	38 (11, 24)	+ Inoculum	8.5
	Yield with inoculum **(tonne ha^{-1})**	2.7 (26)[a]		
	Corn price **(USD tonne^{-1})**	230 (26)	Control	
	Control Yield **(tonne ha^{-1})**	1.3 (26)		

[a]*Yield with inoculum calculated by assuming that relative growth rate penalties translate 1:1 into yield penalties. We assume the worst-case scenario for the use of nitrogen-fixing maize by applying the largest relative growth rate penalty observed (12.8% penalty at seedling stage).*

[b]*Control yield value taken from a report on a plot that had not had fertilizer amended for 2 years* (68).

[c]*Taken from average fertilizer application needed to achieve optimal yield across a range of SSA sites in the mechanistic and empirical models presented in Bonilla-Cedrez 2021* (26)

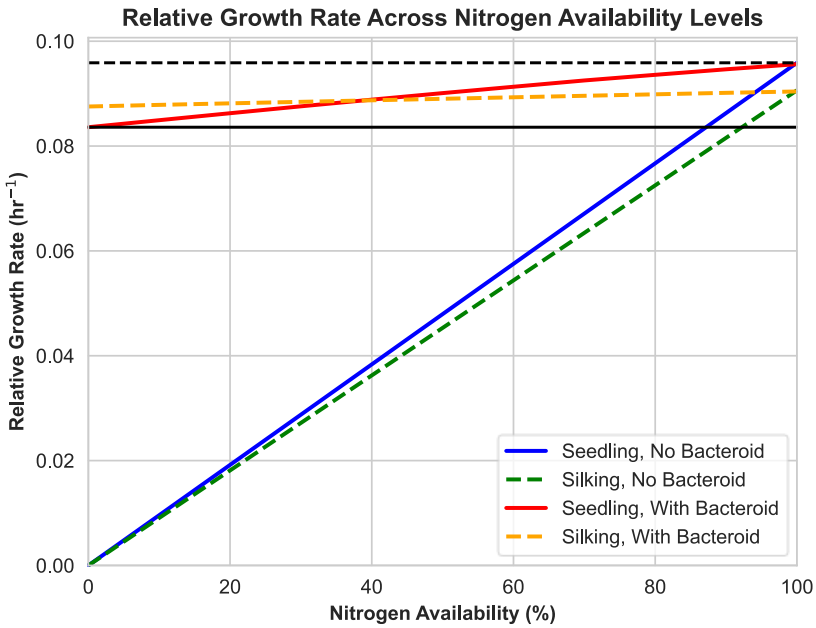

Figure 1: Relative Growth Rate of *Z. mays* across a range of nitrogen availability levels for models with biomass representative of maize seedlings and maize plants at silking. Solid lines represent growth predictions when using the seedling biomass equation and dashed lines represent growth when using the silking biomass equation. The dashed black line represents the maximum growth rate of the non-nodulated model when it receives all its nitrogen from the soil. The solid black line represents the maximum growth rate of the nodulated model when it fully relies on N_2-fixation. For the relative growth rates of the models with biomass representative of the C:N ratios of maize plants at the jointing stage, see **Figure S4**.

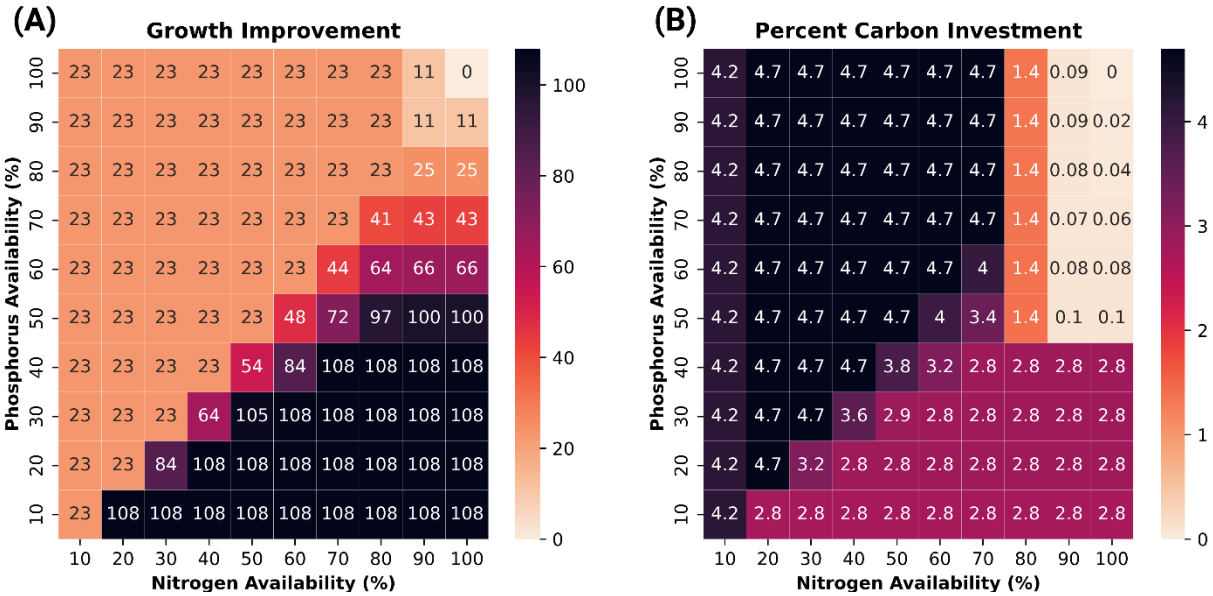

Figure 2: Growth improvement and carbon investment predictions when comparing with- and without-AMF models when maize biomass is representative of seedling C:N ratios. **(A)** Percentage growth improvement of with-AMF vs. without-AMF models of maize as a function of phosphorus and nitrogen availability. **(B)** Predicted carbon investment in AMF as a percentage of net CO_2 assimilation in the plant as a function of phosphorus and nitrogen availability. For plots of growth improvement and carbon investment when the plant biomass is representative of plants at the jointing or silking developmental stages, refer to **Figures S5-6**.

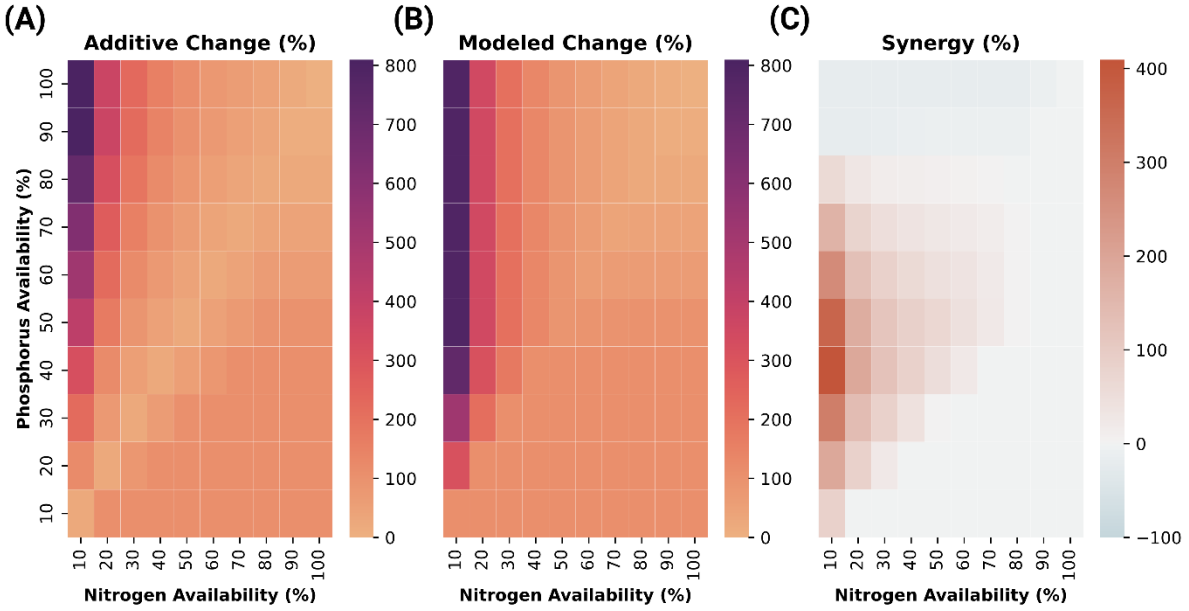

Figure 3: Additive and modeled RGR benefits and calculated synergies and antagonisms in the *Zea mays*, *Bradyrhizobium diazoefficiens*, *Rhizophagus irregularis* three-species system when the maize model's biomass is representative of seedling C:N ratios. **(A)** Sum of relative growth rate changes from adding *B. diazoefficiens* or *R. irregularis* to the *Z.* mays model under given nitrogen and phosphorus availabilities. **(B)** Modeled relative growth rate changes in the three-species model. **(C)** Synergy and antagonism between *B. diazoefficiens* and *R. irregularis* quantified as the difference between the modeled and additive growth rate changes. For the additive change, modeled change, and synergy when the maize model's biomass is representative of plants at the jointing or silking stages, refer to **Figures S7-8**.