

REVIEW

Fundamental Soil Science

Tripartite interactions among free-living, N-fixing bacteria, arbuscular mycorrhizal fungi, and plants: Mutualistic benefits and community response to co-inoculation

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Abstract

Interactions between arbuscular mycorrhizal (AM) fungi and free-living nitrogen fixers (FLNF) occur in the rhizosphere where they can enhance plant nutrient acquisition, impact plant growth, and affect soil processes. Tripartite mutualism commonly occurs between nodule-forming plants, symbiotic diazotrophs, and AM fungi, and can occur between non-nodulating plants, FLNF, and AM fungi. However, information on the extent of, and controls on, tripartite mutualism in non-nodulating plant systems is limited to a small number of crop plants and culturable microbial inoculum, mostly in greenhouse growing conditions. We conducted a systematic literature review to synthesize the current understanding of the responses of plants, AM fungi, and FLNF to co-inoculation, as well as the conditions affecting tripartite mutualism and the magnitude and range of benefits conferred. Our review shows that plants generally benefit from co-inoculation with AM fungi and FLNF taxa, but benefits are highly variable and context dependent, ranging from 94% *reduction* in plant shoot biomass to 255% *increase* in total plant biomass. Additionally, the presence of AM fungi can increase abundance of FLNF and the presence of FLNF can increase AM fungal root colonization, but these responses also vary widely. Major factors influencing variation in response to co-inoculation by all organisms include plant phenology/age, soil type and nutrient availability, and partner pairing. There is potential for leveraging these tripartite mutualisms to improve plant productivity and soil microbial function, but successful application is more likely with a thorough

Abbreviations: AM, arbuscular mycorrhizal; CFU, colony forming unit; FLNF, free-living nitrogen fixers; LRR, log response ratio; PGPM, plant growth-promoting microorganisms.

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understanding of the environmental and mechanistic controls on these relationships and testing of field-scale implementation.

1 | INTRODUCTION

Microbial activity is a critical component of soil function. Plant roots mediate microbial activity by exuding carbon (C) and chemical signals into the rhizosphere (Philippot et al., 2013). Plant growth-promoting microorganisms (PGPM) trigger hormonal stimulation, improve defense against pathogens, and increase nutrient uptake by associated plants (Berg, 2009). PGPM can also stimulate growth and function of other rhizobiota by mobilizing nutrients and triggering increased root exudation (Johansson et al., 2004). Two key groups of plant growth promoting microorganisms are mycorrhizal fungi and N-fixing bacteria.

Arbuscular mycorrhizal (AM) fungi are intercellular, microscopic, obligate mutualists that colonize the roots of over 72% of land plants (Brundrett & Tedersoo, 2018; van der Heijden et al., 2015). They rely on living plants exclusively for all C substrates (Smith & Read, 2008). In exchange for C, AM fungi mobilize nutrients and resources into plant roots, especially phosphorus (P), nitrogen (N), and water, via intracellular nutrient exchange interfaces called arbuscules (Bücking & Kafle, 2015; Hui et al., 2022; Smith & Read, 2008; Wipf et al., 2019). Benefits conferred from AM fungi to the plant are often greater where nutrient availability or water access is limited (Bagyaraj et al., 2015; Begum et al., 2019; Johnson et al., 1997), due to the ability of AM fungal hyphae to forage water and limit nutrients like P from outside the rhizosphere.

N-fixing prokaryotes display various interactions with plants, including (1) non-nodule forming endophytes and epiphytes living in or on plant tissue, like many *Burkholderia* species (Pal et al., 2022), (2) endophytic and symbiotic with the plant, housed in root nodules such as in the legume-*Rhizobia* symbiosis (Afkhami et al., 2021), and (3) free-living and loosely associated with plant roots in the rhizosphere (free-living nitrogen fixers [FLNF]), including many *Azospirillum* and *Azotobacter* spp. (Smercina et al., 2019). In this review, we focus primarily on rhizospheric interactions between FLNF, AM fungi, and plants to elucidate mutualistic interactions beyond the well-studied plant and AM fungal symbioses with nodule-forming N-fixers. FLNF can contribute significantly to plant N uptake, but contributions vary widely and fluctuate episodically (Roley et al., 2019). It is also unclear whether the exchange of N between FLNF and plants is active or passive (Roley, 2021), and the mechanisms that facilitate nutrient exchange between AM fungi and FLNF, or FLNF and plants, remain unknown.

Since the discovery of AM fungi and N-fixing bacteria in the 1800s, researchers have examined relationships between plants and these microorganisms. Specific interest in relationships between AM fungi, FLNF, and plants began in the late 1970s (Bagyaraj & Menge, 1978) after the existence of tripartite symbiosis between AM fungi, *Rhizobium* spp., and leguminous plant hosts was confirmed (Abbott & Robson, 1978; Smith & Daft, 1977). Studies performed in the mid-1980s focused on a variety of plant responses to co-inoculation with different AM fungal consortia and culturable, common, and FLNF species, especially *Azospirillum brasilense*, mostly in greenhouse-based experiments (Figure 1; Table S1).

Early studies found that co-inoculation with AM fungi and FLNF was often associated with an increase in some metric of plant growth, but that the scale and magnitude of plant benefits were variable. These early studies infrequently reported responses of AM fungi and FLNF to co-inoculation, but occasional reports of microbial abundance showed increases in AM fungal root colonization or FLNF abundance (Table S1). Although initial studies of AM fungal-FLNF-plant relationships involved a wide diversity of plants, in the 1990s, the focus turned toward agriculturally and economically important grass species, especially wheat (*Triticum aestivum*) and, later, corn (*Zea mays*) and rice (*Oryza sativa*). This was partially in response to studies revealing that grasses can acquire a significant amount of N from associative N-fixation by FLNF (reviewed by Boddey and Dobereiner [1988]). Tripartite interactions are deemed mutualistic when all three organisms benefit from the interaction. For example, tripartite interactions among non-nodulating plants, AM fungi, and FLNF can improve plant yield (e.g., Barea et al., 1983; Panwar, 1991, 1993; Sharma et al., 2001; Singh et al., 2011) and increase AM fungal root colonization (e.g., Barea et al., 1983; Behl et al., 2007; Mar Vázquez et al., 2000; Sala et al., 2007; Sharma et al., 2001). Tripartite relationships have been leveraged in leguminous plant systems to replenish N to the soil in rotation with non-nodulating crops as part of sustainable agriculture management plans (Ananda et al., 2022), suggesting potential for similar applications in non-nodulating plant systems. However, the tripartite mutualism between non-nodulating plants, AM fungi, and FLNF is much less consistent and more difficult to manage due to the facultative nature of the FLNF-plant and FLNF-AM fungi relationship (Figure 2).

The hypothesized interactions responsible for tripartite benefits in AMF-FLNF-plant mutualism hinge around nutrient exchange among the three organisms. Plants allocate C-rich compounds to roots and into the rhizosphere,

supporting both AM fungi and FLNF (Figure 2A). In exchange for plant C, AM fungi increase plant-available P and N by foraging nutrients from beyond the rhizosphere (Figure 2A,B). FLNF transform inert dinitrogen gas to a bioavailable form via N-fixation (i.e., $N_2 \rightarrow NH_4^+$, Figure 2A,B). However, specific mechanisms of exchange and plant-microbe signaling are largely unclear and require further investigation.

To apply these mutualisms to sustainable agriculture and ecological restoration, a more mechanistic understanding of environmental controls on tripartite mutualism is essential. We systematically examined 47 studies on co-inoculation of non-nodulating plants with AM fungi and FLNF and summarized current knowledge in three key areas: (1) the magnitude and type of benefits conferred to plants, AM fungi, and FLNF as a result of co-inoculation; (2) contextual dependency on environmental conditions, which affect the magnitude and scale of benefits conveyed; and (3) similarities and gaps across study designs investigating these relationships.

2 | MATERIALS AND METHODS

2.1 | Study eligibility criteria

Studies included in this review were selected with Google Scholar and Web of Science using search terms such as

Core Ideas

- Co-inoculation with arbuscular mycorrhizal (AM) fungi and free-living nitrogen fixers (FLNF) most consistently benefits plants by increasing total biomass and yield.
- Co-inoculation with FLNF consistently increases AM root colonization in both cereal and non-cereal plants.
- FLNF response to co-inoculation is underreported, but evidence suggests abundance increases in non-cereal plants.
- Overall responses to co-inoculation by all organisms are generally positive, but highly variable.
- Variability in response to co-inoculation largely depends on partner pairing, resource availability, and plant age.

free-living N-fixation, associative N-fixation, rhizosphere diazotrophs, AMF, arbuscular mycorrhiza, co-inoculation, and non-nodulating plants. The literature search was conducted between the fall of 2019 and July of 2021. Articles returned from these searches were manually curated for inclusion under the following criteria: (1) the study included

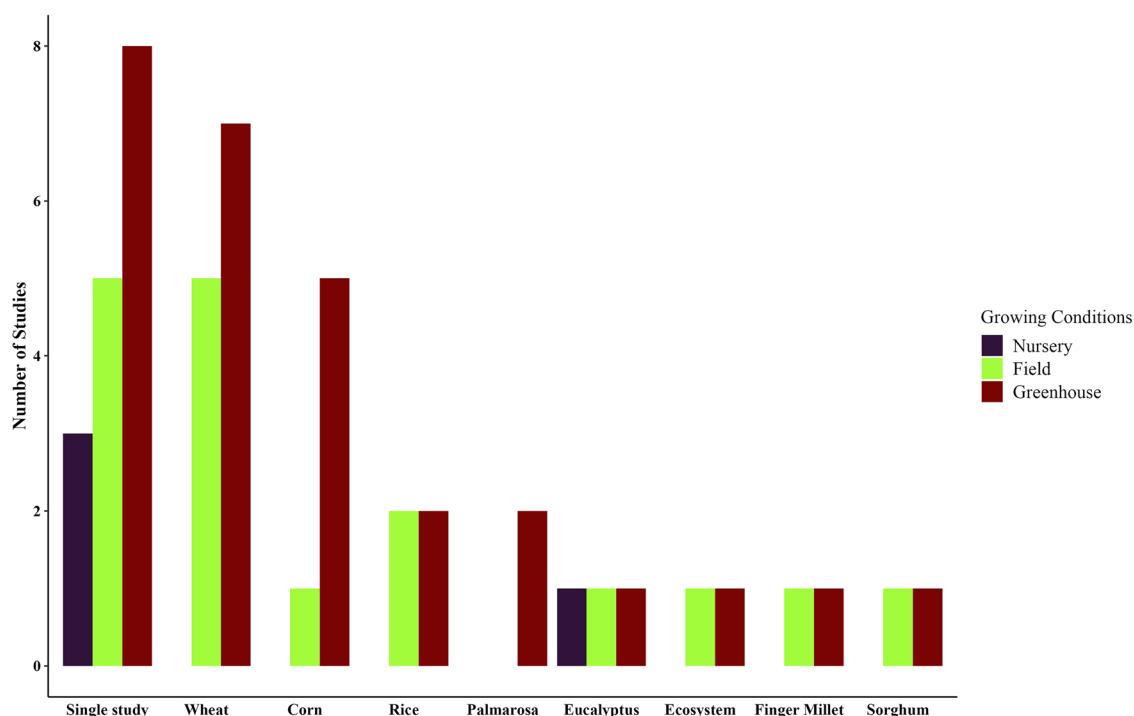


FIGURE 1 Plants used across 47 studies (1978–2020) investigating interactions between arbuscular mycorrhizal (AM) fungi, free-living nitrogen fixers (FLNF), and host plants grown under experimental greenhouse, field, or nursery conditions. Plant species included in only one study are represented together here in the “single study” column comprising 42% of total studies, and two studies on whole plant communities are represented together in the “Ecosystem” column.

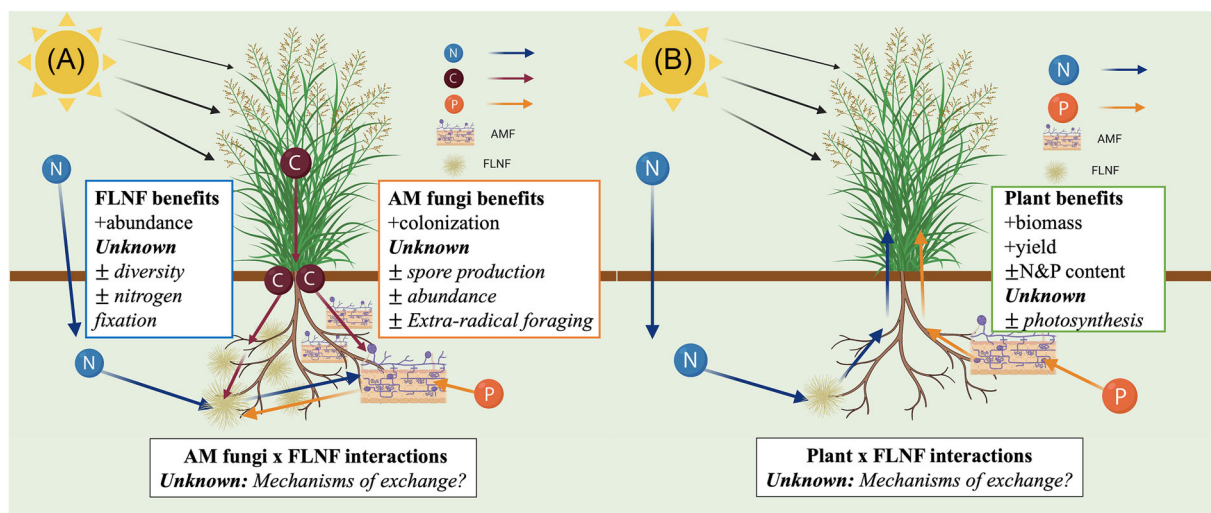


FIGURE 2 Conceptual diagram showing the proposed mechanisms of potential N, P, and C exchange as a result of tripartite mutualism, and demonstrated and unknown potential benefits conferred to (A) free-living nitrogen fixers (FLNF) bacteria and arbuscular mycorrhizal (AM) fungi communities, and (B) host plants. Figure created using BioRender.com.

interactions between plant(s), at least one taxon of AM fungi and at least one taxon of FLNF in the rhizosphere; (2) the study did not include a legume or actinorhizal plant species (because we focus here on root-associative, rather than endophytic N-fixers); and (3) the study reported responses of at least one group of target organisms (i.e., AM fungi, FLNF, or plants) to co-inoculation with AM fungi and FLNF in the rhizosphere.

Published papers vary in quality and we aimed to focus on well-designed, well-replicated studies in our interpretation while still incorporating contributions from smaller scale papers. To do so, we ranked each study against seven objective criteria designed to assess its scientific rigor and reproducibility. Criteria used in this assessment included: number of treatments and replicates per treatment, the inclusion of proper controls for each treatment involved, number of AM fungal taxa used as inoculum, number of FLNF taxa used as inoculum, number and type of response measurements reported, and whether the study recorded responses to co-inoculation by more than one of the three groups of organisms involved. To assess reproducibility, each study was assessed based on whether key methodological areas were described in enough detail to easily replicate soil type, growing conditions, inoculation strategy, measurements, and data analysis. This qualitative assessment resulted in 11/47 studies (23%) ranking 5/7 or higher, and 31/47 (66%) of studies ranking 4/7 or lower. Only one study, Welsh et al. (2010), fulfilled all seven objective quality criteria. Studies ranking 5 or higher were given more consideration relative to average results in the discussion.

Additionally, a funnel plot analysis was performed to visualize potential publication bias in the studies analyzed in this systematic review. A funnel plot was generated with the “metafor” package using R statistical software (R Core

Team, 2022; Figure S1) by plotting the sample size (replicate number) on the Y axis against the mean LRR (log response ratio, see next paragraph for equation) of each measurement reported for each study.

2.2 | Collection and comparison of data from individual studies

Data were extracted from figures and tables presented in the publications or in the Supporting Information. The tool Web-PlotDigitizer 4.6 (<https://apps.automeris.io/wpd/>) was used to extract data from figures when numerical data were not available. We calculated effect size as the LRR (Hedges et al., 1999; Rubin et al., 2017):

$$\text{LRR} = \ln(V_i/V_c)$$

where V_i is the treatment (co-inoculated) mean and V_c is the single-inoculate control mean; whichever single inoculate (AM fungi or FLNF) performed best in each study is observed. The LRR is commonly used in ecological meta-analyses because it normalizes differences in units and scales reported across studies and reduces data skewness (Rubin et al., 2017). LRR values were converted to percentage change (%Δ) using the following formula (Rubin et al., 2017):

$$\% \Delta = 100 \times (\exp. (\text{LRR}) - 1).$$

We calculated LRR and percentage change for the eight most reported plant and microbial response variables: total plant biomass, root biomass, shoot biomass, plant yield (e.g., total grain or fodder harvested), plant N, plant P, percentage root colonization by AM fungi, and number of FLNF colony

forming units (CFUs). We used a single tailed *t*-test to determine if mean effect size values were significantly different than zero.

Plant benefits resulting from co-inoculation are often reported in comparison to uninoculated controls. This can be problematic because the addition of AM fungi or FLNF alone can have a larger effect on plant growth than when added as co-inoculates (Bauer et al., 2012; Kamali & Mehraban, 2020; Miyauchi et al., 2008; Pacovsky et al., 1985; Sala et al., 2007). Here, we have calculated LRR and report %Δ based on responses to co-inoculation relative to whichever single-inoculate control performed better in each metric evaluated (Table 1; Table S1). This resulted in treatment effect sizes in this review that differ, in some cases, from those in the original publication, but allow us to specifically address the interactions among the three target groups (FLNF, AMF, and plants).

Positive-response publication bias has some influence on our results according to a funnel plot analysis (Figure S1). Studies with fewer replicates used in this analysis tended to have positive LRR values and higher variability than studies with more replicates, which tended to have more neutral LRR values. Positive-results bias occurs when studies with positive results are more likely to be published than those with negative or null results. High impact journals tend to select for publications with novel or positive study outcomes, which can further inflate overall bias toward overestimation of positive results and increased effect sizes (Joobert et al., 2012). In the case of FLNF-AMF-plant mutualism, a positive-results bias would overgeneralize the effectiveness and associated benefits conferred by co-inoculation, although it is difficult to estimate how frequently this occurs (Lortie et al., 2007). We cannot fully resolve issues of positive-results bias, but we did attempt to minimize this bias. We considered all results derivable from published data, including negative and null results, and considered the best performing, single inoculate of either AM fungi or FLNF as the control in our systematic review. Additionally, we normalized reported effects using LRR and compared all studies using %Δ. Finally, we compared important qualitative measures such as number and taxonomy of inoculates used, growing conditions, and plant species to identify key knowledge gaps.

3 | RESULTS AND DISCUSSION

3.1 | Plant responses to co-inoculation with AM fungi and FLNF

When rhizospheres are co-inoculated by AM fungi and FLNF, some metric of plant growth and/or nutrient uptake is generally improved (Figure 3A; Table 1). Overall, response metrics increased by an average of 3% (LRR $p > 0.05$) in

TABLE 1 Mean log response ratio (LRR) and percentage change (%Δ) ± standard error in commonly reported measurements of plant, arbuscular mycorrhizal (AM) fungi, and free-living nitrogen fixers (FLNF) responses to co-inoculation from 35 studies with extractable data.

	Plant growth response to co-inoculation			AMF root colonization		FLNF cell count	
	Total biomass	Root biomass	Shoot biomass	Yield	Plant P	Plant N	AM fungal root colonization
LRR	0.27 ± 0.07*	0.10 ± 0.03*	0.08 ± 0.06	0.21 ± 0.04*	0.11 ± 0.03*	-0.02 ± 0.04	0.23 ± 0.03*
Range (%Δ)	-18.8 to 254.5	-50 to 88.9	-93.5 to 74.6	-7.1 to 174.3	-39.1 to 100	-64 to 66.1	-39.4 to 128.7
Mean (%Δ)	42 ± 5.1	13 ± 1.7	14.9 ± 2	28.8 ± 3.1	15.1 ± 2	3 ± 1.6	30.7 ± 3
Median (%Δ)	14.1	9.9	16.7	11.5	9.3	4.5	22.2
Number of reporting studies	13 (37%)	18 (51%)	18 (51%)	12 (34%)	15 (43%)	13 (37%)	21 (60%)
							6 (17%)

Note: LRR and %Δ compare measurements in plants co-inoculated with AM fungi and FLNF (treatment) with those inoculated with AM fungi or FLNF alone (control). LRR values in bold are different than zero ($p \leq 0.05$) according to a single-tailed *t*-test. Italicized values indicate a negative response to co-inoculation.

Abbreviation: CFUs, colony forming units.

*Significant at the 0.05 probability level.

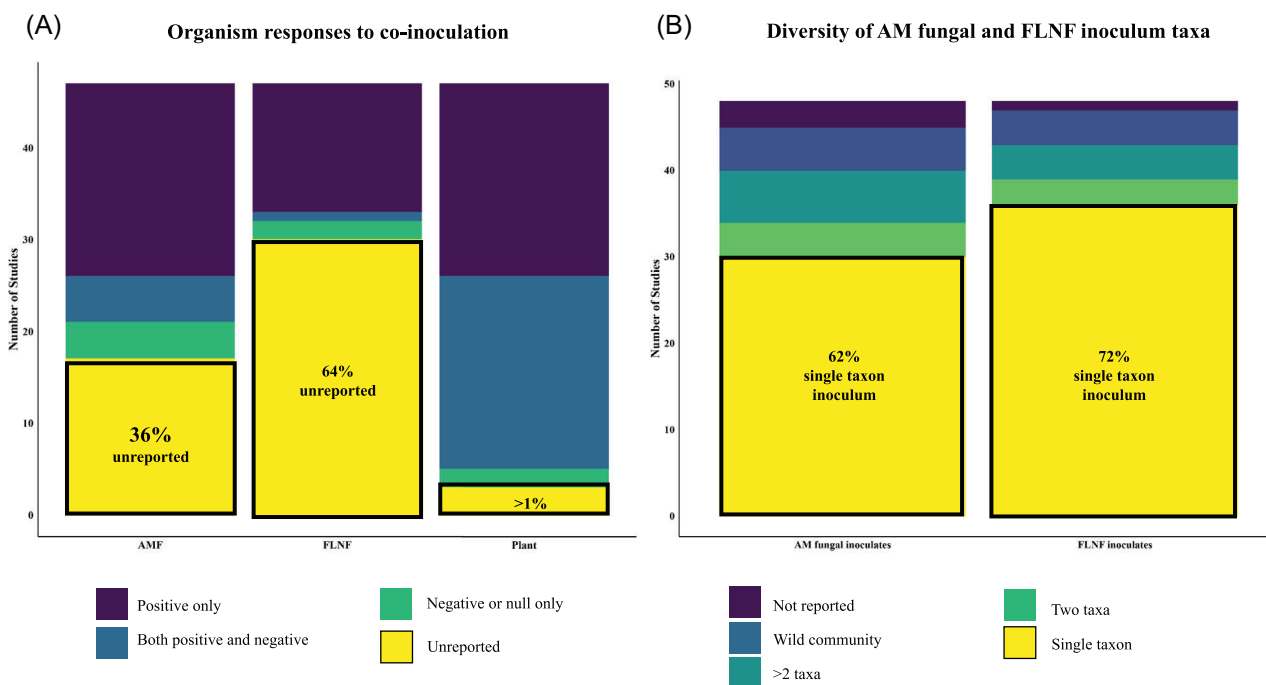


FIGURE 3 (A) Number of studies showing positive, negative, or null responses to co-inoculation by arbuscular mycorrhizal fungi (AMF), free-living nitrogen fixers (FLNF), and plants. Percentages in black show percentage of studies that did not report responses of AM fungi, FLNF or plants. (B) Number of studies using only a single taxon of AM fungi or FLNF for inoculation experiments versus more complex communities of both groups. Percentages in black show percentage of studies that only used a single taxon of either AM fungi or FLNF as inoculum.

plant N content and 42% (LRR $p < 0.05$) in total plant biomass relative to single inoculates of either AM fungi or FLNF (whichever performed best) in each study (Figure 3A; Table 1). However, plant growth responses to inoculation were highly variable (Table 1), and all growth metrics had at least one study where the effects of co-inoculation were negative. Both mean shoot biomass and Plant N LRR values were not statistically different than zero ($p > 0.05$), indicating weak overall response to co-inoculation (Table 1). Additionally, most studies that looked at plant growth response across more than one nutrient application, partner pairing combination, or soil type reported both positive and negative or neutral plant growth responses from the same study (Figure 3A), illustrating the importance of environmental context to tripartite relationships. A higher proportion of studies report greater positive plant responses to co-inoculation in non-cereal versus cereal plants under both field and greenhouse conditions, despite a higher number of repeat studies focusing on cereal crops (Figure 1; Table 2; Table S1).

However, due to low replication, many of these positive LRR values associated with non-cereal grain studies are not significantly larger than zero (Table 2). In addition to the response metrics presented in Tables 1 and 2, other positive plant responses to co-inoculation can include increased photosynthesis (Koocheki et al., 2008; Panwar, 1991), stomatal conductance (Ruíz-Sánchez et al., 2011), and root exploration (Mar Vázquez et al., 2000; Muthukumar et al., 2001; Muthukumar & Udaiyan, 2006; Santhosh et al., 2018;

Sultana & Pindi, 2012; Thamizhiniyan & Panneerselvam, 2011; Table S1). Co-inoculation generally has a smaller effect on total plant biomass (mean 42.1% increase, LRR $p < 0.05$; Table 1) and yield (mean 28.8% increase, LRR $p < 0.05$, Table 1) than does standard N and P fertilizer addition to most crops, which can range up to 136% total biomass (H. Li et al., 2023) and 40%–60% average yield increase (Stewart et al., 2005). However, increases in plant biomass and nutrient content in response to co-inoculation can compare to, or exceed, those produced from standard fertilization application in some species and under some growing conditions (Barea et al., 1983; Reyes et al., 2020; Table 1), especially in cereal crops grown under greenhouse conditions (average yield 59.9% change, LRR $p < 0.05$, Table 2). For example, co-inoculation can increase plant yield and N content in the forage grass *Urochloa decumbens* at scales equivalent to a range of N fertilizer application levels (Reyes et al., 2020), suggesting the potential for such mutualisms to reduce the need for high volumes of fertilizer application to certain crops, under certain environmental conditions (Casler, 2022; Fukami et al., 2016; Reyes et al., 2020). However, outcomes of co-inoculation on plant yield and growth vary widely (Tables 1 and 2), making it difficult to predict whether they will be effective at larger scales.

Occasionally, no effect on plant growth is observed but other benefits of co-inoculation are evident. For example, some corn plants increased in tissue P concentration without changes in plant growth (Gryndler & Hřelová, 1998), a

TABLE 2 Comparison of responses by cereal crop plants versus non cereal crop plants, arbuscular mycorrhizal (AM) fungal, and free-living nitrogen fixers (FLNF) grown under field or greenhouse (including nursery) conditions ($\pm$ standard error). Percentage change (% Δ) in the commonly reported measurements of plant, AM fungi, and FLNF responses to co-inoculation with AM fungi and FLNF (treatment) versus inoculated with AM fungi or FLNF alone (control) across 35 studies with extractable data.

Response metric	Field				Greenhouse			
	Cereal	Number of studies	Non-cereal	Number of studies	Cereal	Number of studies	Non-cereal	Number of studies
Total biomass (% Δ)	17.7 $\pm$ 4.4	3	29.9 $\pm$ 15.1	2	73.4 $\pm$ 25	5	-13 $\pm$ 6.9	3
LRR	0.15 $\pm$ 0.04		0.24 $\pm$ 0.15		0.43 $\pm$ 0.13		-0.01 $\pm$ 0.07	
Root biomass (% Δ)	12.3 $\pm$ 4.5	2	88.9 $\pm$ 0	1	5.6 $\pm$ 3.2	8	15.6 $\pm$ 5.3	7
LRR	0.11 $\pm$ 0.04		0.64 $\pm$ 0		0.05 $\pm$ 0.03		0.17 $\pm$ 0.05	
Shoot biomass (% Δ)	28 $\pm$ 11.3	2	66.7 $\pm$ 0	1	8.1 $\pm$ 3.6	8	17.7 $\pm$ 7.2	7
LRR	0.24 $\pm$ 0.09		0.51 $\pm$ 0		0.06 $\pm$ 0.03		0.07 $\pm$ 0.14	
Yield (% Δ)	7.5 $\pm$ 1.9	5	22.8 $\pm$ 5.6	3	59.9 $\pm$ 18.7	4	0	0
LRR	0.07 $\pm$ 0.02		0.23 $\pm$ 0.06		0.4 $\pm$ 0.12		0 $\pm$ 0	
Plant P (% Δ)	8.7 $\pm$ 0	1	10.8 $\pm$ 2.6	2	5.6 $\pm$ 11.5	5	24.9 $\pm$ 7.2	7
LRR	0.08 $\pm$ 0		0.08 $\pm$ 0.09		0.05 $\pm$ 0.03		0.19 $\pm$ 0.06	
Plant N (% Δ)	66.1 $\pm$ 0	1	9.1 $\pm$ 6.5	2	-10.3 $\pm$ 5.8	5	10.4 $\pm$ 3.9	5
LRR	0.51 $\pm$ 0		0.07 $\pm$ 0.06		-0.16 $\pm$ 0.08		0.09 $\pm$ 0.04	
AM fungal root	18.3 $\pm$ 4.3	2	52.2 $\pm$ 13.6	3	22.5 $\pm$ 7.2	8	41.6 $\pm$ 7	8
LRR	0.16 $\pm$ 0.03		0.37 $\pm$ 0.09		0.19 $\pm$ 0.06		0.32 $\pm$ 0.06	
FLNF CFUs (% Δ)	0	0	27.6 $\pm$ 8.1	1	32.7 $\pm$ 22.6	2	54.5 $\pm$ 18.8	3
LRR	0 $\pm$ 0		0.23 $\pm$ 0.06		0.25 $\pm$ 0.15		0.33 $\pm$ 0.11	

Note: Mean LRR values in bold are different than zero ($p \leq 0.05$) according to a single-tailed t -test.

Abbreviations: CFUs, colony forming units; LRR, log response ratio.

common plant response to inoculation with AM fungi alone (H. Li et al., 2006, 2008; Smith et al., 2003). Similarly, co-inoculation with the AM fungus *Rhizophagus irregularis*, and FLNF bacterium *Azotobacter vinelandii* had little effect on grain yield or N uptake but did increase wheat root exploration in a field experiment (Cortivo et al., 2018). Plant benefits of co-inoculation are commonly further increased when other plant growth promoting microorganisms are included in the inoculum with AM fungi and FLNF (Kumar et al., 2015; Muthukumar & Udaiyan, 2006; Sultana & Pindi, 2012). This is due to a wide range of microbial plant-growth promoting activities, including nutrient mobilization and biological control against pathogens (Pii et al., 2015), highlighting the importance of whole community interactions in beneficial outcomes. Studies included here that were identified as having high scientific rigor consistently showed greater positive plant response when rhizosphere microbial diversity was experimentally expanded beyond AM fungal and FLNF taxa and reiterate that positive plant responses to co-inoculation are not always increased relative to plants singly inoculated with AM fungi or FLNF alone.

On aggregate, high-quality studies support the general conclusion that co-inoculation with AM fungi and FLNF improve some metric of plant growth and/or nutrient uptake. However, the more rigorous studies tended to measure a wider variety of responses to co-inoculation and test more param-

eters affecting tripartite relationships. The added granularity of these studies also identifies observed exceptions to broader general conclusions and highlights areas where more research is needed to determine optimal conditions for successfully applying these relationships. When plants did not respond positively to co-inoculation, a few specific controls stood out. These include changes in plant phenology or age (Bagyaraj & Menge, 1978; Muthukumar et al., 2001; Welsh et al., 2010), differences in microbial partner pairing (Mar Vázquez et al., 2000; Muthukumar et al., 2001; Rao et al., 1985; Subba Rao et al., 1985), soil type (Pacovsky et al., 1985), or plant cultivar (Miyauchi et al., 2008). For example, a field study on the effect of indigenous populations of AM fungi and FLNF on growth and nutrient content of salt meadow cordgrass (*Spirilla patens*) showed significant increases in biomass only during vegetative and reproductive phenological phases, and plant P and N content significantly decreased during vegetative growth in co-inoculated plants (Welsh et al., 2010).

3.2 | Interactions between AM fungi and FLNF in the rhizosphere

AM fungal and FLNF responses to co-inoculation are variable, but some consistent positive responses by both groups of microorganisms have been observed. Positive responses to

co-inoculation in these groups may be due to high requirements of P for N-fixation by FLNF (O'Hara, 2001) and high N demand for the production of AM fungal extraradical hyphae (Hodge et al., 2010). AM fungi generally respond to co-inoculation by increasing root colonization (Table 1, 30.7% increase, LRR $p < 0.05$), even when FLNF communities do not appear to be affected by AM fungi, especially in non-cereal plants (Reyes et al., 2020; Sala et al., 2007; Will & Sylvia, 1990; Table 2). Positive growth responses of AM fungi to co-inoculation with FLNF in the rhizosphere could be attributed to increased local N availability due to fixation, combined with increased root exudation of C by plants (Canarini et al., 2019). However, mechanisms of nutrient exchange between AM fungi and FLNF remain poorly understood and highly speculative.

FLNF abundance as measured in CFUs consistently shows an increase in population size in response to co-inoculation (Table 1, 46.3% change, LRR $p < 0.05$; Bagyaraj & Menge, 1978; Behl et al., 2007; Bellone & de Bellone, 1995; Mar Vázquez et al., 2000; Mishra et al., 2008; Miyauchi et al., 2008; Muthukumar et al., 2001; Muthukumar & Udaiyan, 2006; Raimam et al., 2007; Sharma et al., 2001), although this response is infrequently measured (Figure 3B). This boost in FLNF population and/or fixation could result from increased P availability via AM fungal extraradical hyphae. AM fungi can increase P availability by foraging outside the rhizosphere for bioavailable P that is transferred to the plant via arbuscules in exchange for C (Ezawa et al., 2002). AM fungi-foraged P may become directly available to rhizobacteria via hyphal exudation (Zhang et al., 2022) or by transporting bacteria in biofilm on foraging hyphae (Jiang et al., 2021). Two common and culturable rhizosphere FLNF genera, *Azospirillum* and *Azotobacter*, are especially prone to increasing in abundance when co-inoculated with AM fungi (Miyauchi et al., 2008; Muthukumar et al., 2001; Welsh et al., 2010). This suggests AM fungi can increase abundance of certain culturable FLNF genera, and that some taxonomic groups of FLNF might interact more positively with AM fungi than others.

Although we observed some general trends in AM fungi and FLNF response to co-inoculation, we note that only a limited number of response measurements are commonly reported, and clear mechanisms facilitating these apparent positive interactions remain largely hypothetical (e.g., direct vs. passive exchange of N and P between FLNF, AMF, and plants). Additionally, when more parameters are tested in studies with high scientific rigor, these general trends become complicated by variation associated with plant phenology, soil composition, and partner pairing (Bagyaraj & Menge, 1978; Mar Vázquez et al., 2000; Miyauchi et al., 2008; Muthukumar et al., 2001; Pacovsky et al., 1985; Rao et al., 1985; Subba Rao et al., 1985; Welsh et al., 2010). To better understand and predict the ecological relevance of co-inoculation, and leverage tripartite relationships in restoration and agri-

culture, we suggest that future studies address extraradical exploration by AM fungi, FLNF-derived N in fungal hyphae, N-fixation by FLNF, photosynthetic rate of plants, and proportion of FLNF-derived N in plant biomass. These specific measurements would better address the mechanisms by which organisms benefit (or not) from tripartite interactions.

3.3 | Field application

Scaling up successful greenhouse inoculation methods for field application is key to identifying how beneficial plant-microbe relationships can be used in agriculture and restoration. Currently, field-based experiments are conducted less frequently than greenhouse experiments (Figure 1; Table 2), but when field and greenhouse experiments are paired or directly compared, they can yield similar results (Cortivo et al., 2018; Reyes et al., 2020; Santhosh et al., 2018; Sheng et al., 2019; Siddaram et al., 2017). For example, in concordance with greenhouse studies, co-inoculation under field conditions can increase plant biomass, yield, and/or nutrient uptake, sometimes at magnitudes comparable to fertilizer addition (Bama & Ramakrishnan, 2010; Behl et al., 2007; Koocheki et al., 2008; Mishra et al., 2008; Reyes et al., 2020; Santhosh et al., 2018; Sharma et al., 2001; Siddaram et al., 2017; Solovyeva et al., 2011; Sridevi & Ramakrishnan, 2010; Thamizhiniyan & Panneerselvam, 2011). We found that, on average, co-inoculation increased total biomass and root biomass in cereal crops and increased yield in both cereal crops and non-cereal crops (Table 2). However, these responses are inconsistent, and outcomes from field experiments can vary with environmental factors that are more difficult to control in field settings. For example, biomass, plant P, plant N, and AM fungal colonization increase with co-inoculation in some soil types but decrease in others (Pacovsky et al., 1985). Additionally, not all plant species or cultivars respond the same way to co-inoculation even under the same field environments (Barea et al., 1983; Behl et al., 2007).

AM fungal communities can also respond positively to co-inoculation under field conditions. In perennial C4 grasses (e.g., *Panicum maximum* and *Urochloa decumbens*) and wheat, AM fungal spore counts (Mishra et al., 2008; Reyes et al., 2020) and root colonization increased with co-inoculation (Behl et al., 2007; Reyes et al., 2020; Sharma et al., 2001). FLNF responses to co-inoculation in the field are rarely reported, but there is some evidence that co-inoculation can increase FLNF abundance and diversity under field conditions as well (Behl et al., 2007; Mishra et al., 2008; Welsh et al., 2010; Zarea et al., 2009).

A few key factors are currently inhibiting advancement of large-scale, field application of AM fungi-FLNF co-inoculations. First, it is impossible to control all confounding

variables in a field setting, and a lack of consistent responses to co-inoculation across greenhouse experiments further limits the ability to identify important controls in the field. Second, methods to ensure adequate survival, colonization, and sufficient addition of inoculum across large areas have not been well-established. Moreover, the potential to introduce invasive species that might damage indigenous soil microbial communities is poorly understood and warrants further testing (Jack et al., 2020). Finally, the costs of application can be prohibitive, especially with uncertain likelihood of inoculation success.

Before this tripartite relationship can be used as a reliable biofertilizer supplement, some key questions must be addressed. First, can successful greenhouse inoculations be sustainably scaled to the field to produce consistent results? Method development for implementing at the field-scale is needed for application in agriculture and soil restoration. Next, what are the strongest environmental controls on tripartite mutualism in the field? Specifically, environmental conditions improving and reducing the effectiveness of these relationships should be defined. Finally, how can we optimize partner pairing for specific plants and regions of interest? Identification of plant-specific and region-specific beneficial taxa of AM fungi and FLNF that function well together in field settings will help with field application. Subsequently, the potential for microorganisms in applied inoculum to become invasive should be better understood before large scale applications take place.

3.4 | Context dependency

The magnitude and variety of benefits exchanged in the AM fungi–FLNF–plant mutualism varies with context (Table S1). This context dependency appears to be largely driven by partner pairing, plant phenology/age, and resource availability, but is also affected by abiotic factors such as climate, precipitation, and soil pH and texture. Partner pairing is often a strong driver of plant growth response to AM fungal and FLNF rhizosphere communities (Table S1). Some plant species require a more specific consortia of AM fungal and FLNF taxa than others (Emery et al., 2018; Mar Vázquez et al., 2000; Rao et al., 1985; Sala et al., 2007; Sugavanam et al., 2000; Sultana & Pindi, 2012), and certain plant cultivars or successional stages are more responsive to microbial inoculants than others (Behl et al., 2007; Cheeke et al., 2019; Gryndler & Hřselová, 1998). Variation in mycorrhizal traits between AM fungal taxa such as colonization intensity and resource exchange rates between plants and fungi can be important factors in determining which AM fungal taxa convey the greatest benefits (Chaudhary et al., 2022). For example, root biomass increased with co-inoculation in some but not all wheat and corn cultivars in field experiments (Gryndler &

Hřselová, 1998), and Rao et al. (1985) showed that increased biomass and P uptake by pearl millet (*Pennisetum americanum*) only occurred when co-inoculated with combinations of FLNF species *A. brasilense*, and either *Rhizophagus fasciculatus* or *Gigaspora margarita* AM fungal species. Similar plant responses to specific partner pairing combinations were observed in barley (*Hordeum vulgare*; Subba Rao et al., 1985), corn (*Z. mays*; Gryndler & Hřselová, 1998; Mar Vázquez et al., 2000), wheat (*Triticum aestivum*; Behl et al., 2007; Sala et al., 2007), *Eucalyptus* spp. (Karthikeyan & Prakash, 2008; Sugavanam et al., 2000), and cotton (*Gossypium* sp., Sultana & Pindi, 2012). Similarly, FLNF can disproportionately benefit certain plant functional groups, such as perennial grasses and other C4 plants over others (Davies-Barnard & Friedlingstein, 2020; Wewalwela et al., 2020).

In most cases, ideal microbial partners have yet to be identified, or are not readily culturable, so most studies inoculate with only a single species or genus of cultured or commercially purchased AM fungi and/or FLNF taxa (here, 60% and 80% of studies, respectively, Figure 3). Limitations on inoculate diversity potentially lowers inoculation success and makes identification of appropriate partner pairs challenging. Optimal combinations of AM fungi and FLNF consortia likely exist for specific plant species, communities, and ecosystems. The potential for microbial inoculation to serve as effective biofertilizer is due in large part to the fact that soils disturbed by anthropogenic practices, such as conventional farming, are generally depleted of beneficial microbial communities (Köhl et al., 2014; Oehl et al., 2005; Wittwer et al., 2021). Naturally occurring microbial consortia include more diverse pairings of AM fungi, FLNF, and other beneficial rhizobiota that are not commonly represented in inoculation experiments. In agroecosystems, microbial filtrate (Mar Vázquez et al., 2000) or whole soil inoculum derived from conservation or organic agroecosystems may help restore microbial processes in soils depleted by conventional agriculture practices (Gutiérrez Landázuri et al., 2020; Köhl et al., 2014; Wittwer et al., 2021). In the context of ecological restoration, inoculating with microbial communities from a locally adapted, healthy population of the target plant community or ecosystem type is generally more effective at improving plant nutrient acquisition and restoration outcomes than commercial inoculates (Gutiérrez Landázuri et al., 2020; Middleton & Bever, 2012; Wubs et al., 2016). However, for long-term success, the live-soil inoculum must be part of a healthy soil ecosystem, conducive to survival and proliferation of the inoculum, and the inoculation method must be of appropriate scale.

Plant phenology and age can also impact the magnitude of benefits exchanged between plants, AM fungi, and FLNF. Plants can alter the amount of C they exude through roots in response to physical and chemical stimuli, which is often in accordance with a plant's age or phenological phase, thereby

changing their interactions with soil microbiota (Bever et al., 2009; Warembourg & Estelrich, 2001). In response, microorganisms may alter the flow of nutrients to the plant, causing variation in both plant growth response and rhizosphere microbial community structure (Anzuay et al., 2021). These phenological effects can vary between plant growth forms and species. For example, FLNF abundance and N-fixation rates increased in sugar cane during vegetative growth stages, while both fixation rate and FLNF diversity decreased with plant age (de Lima et al., 2021). In switchgrass (*Panicum virgatum*), FLNF community structure was consistent throughout the growing season (B. B. Li et al., 2021), but N-fixation varied with phenological stage (Roley et al., 2018). Thus, benefits of tripartite interactions between plants, AM fungi, and FLNF are potentially inconsistent across time, based on plant-related co-factors influencing when and how much C is allocated to the rhizosphere (Vives-Peris et al., 2019), how much associative N-fixation takes place (Smercina et al., 2019), and the volume of nutrient exchange with AM fungi (Wipf et al., 2019).

Resource availability can have a profound effect on nutrient exchange-based mutualisms. When a resource exchanged in the mutualism is overabundant or sufficiently bioavailable in the soil (here N, P, C, or water), the mutualism can become less valuable to any or all of the partners involved, decreasing the strength of the interaction (Jach-Smith & Jackson, 2018; McKinley, 2019; Smercina et al., 2019). In contrast, when a nutrient or resource is limiting, the mutualism tends to be more beneficial for plants (Bever et al., 2009; Johnson et al., 1997; Warembourg & Estelrich, 2001). In the case of AM fungi–FLNF–plant relations, plants benefit more from co-inoculation under conditions of low N availability and drought stress (Reyes et al., 2020; Ruíz-Sánchez et al., 2011). For example, bamboo (*Bambusa vulgaris*) growth response to co-inoculation in a nursery experiment decreased with fertilizer application (Muthukumar & Udaiyan, 2006), and plants under drought stress increased yield, photosynthesis, and stomatal conduction as a result of co-inoculation (Ruíz-Sánchez et al., 2011). Microbial responses to resource availability can mirror plant responses when co-inoculated. For example, *Azospirillum spp.* population abundance decreased with fertilizer addition in the same bamboo nursery study (Muthukumar & Udaiyan, 2006), and AM fungal colonization and spore production was inversely proportional to N fertilization in *Urochloa decumbense* and rice (*O. sativa*) roots (Reyes et al., 2020; Ruíz-Sánchez et al., 2011).

3.5 | Conclusions

Mutualistic tripartite interactions occur among non-nodulating plants, AM fungi, and FLNF. These interactions can benefit plant growth and/or nutrient status. Benefits of the tripartite relationship can extend to some AM fungal and

FLNF taxa as demonstrated through increased root colonization or abundance, respectively, while functional responses to co-inoculation remain mostly unknown. In some cases, one or more partners are adversely affected by co-inoculation in some way, even when benefitting in other ways. Nutrient and water availability, along with plant phenology or age, can strongly influence the magnitude and outcomes of non-nodulating plant $\times$ AM fungi $\times$ FLNF interactions. The current body of literature suggests potential for the use of mutualistic tripartite relationships as biofertilizer to increase plant productivity and soil microbial function in some but not all ecosystems, nor at all times of the growing season, or between all plant, AM fungal, and N-fixing partners. It should be noted that our funnel plot analysis indicated some positive response bias based on the data used in this study, suggesting that the reported benefits of tripartite relationships might be somewhat inflated toward positive response outcomes for all eight response measurements analyzed (Figure S1).

Important knowledge gaps remain in some key areas, preventing effective and consistent application of biofertilizers development. For instance, we need to assess the benefits to and of broader microbial consortia including non-culturable AM fungi and FLNF, and whether some taxa work better together than others. Promising experimental outcomes from greenhouse settings are infrequently tested in field settings, inhibiting our ability to scale up positive outcomes to field application. Recent research on the hyphosphere has provided more insight into the mechanisms behind exchange of plant C for N, P, and water from AM fungi (Bever et al., 2009; Bücking & Kafle, 2015; Chaudhary et al., 2022; Ezawa et al., 2002; Hui et al., 2022; Jiang et al., 2021; Johansson et al., 2004; Sheng et al., 2019; Wipf et al., 2019; Zhang et al., 2022). However, mechanisms of nutrient exchange between AM fungi and FLNF, and from FLNF to non-nodulating plants remain unclear. Focusing future research on clarifying these key areas of uncertainty will allow the development of more consistent and effective application of this tripartite mutualism as biofertilizer in agroecosystems and in ecological restoration.

AUTHOR CONTRIBUTIONS

Shawnee A. Kasanke: Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing—original draft; writing—review and editing. **Tanya E. Cheeke:** Investigation; methodology; resources; supervision; writing—review and editing. **James J. Moran:** Funding acquisition; methodology; resources; supervision; validation; writing—review and editing. **Sarah S. Roley:** Funding acquisition; methodology; resources; supervision; validation; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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