

# Effects of dual mycorrhizal inoculation on *Pinus strobus* seedlings are influenced by soil resource availability

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## Research Article

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# Abstract

## PURPOSE

Mycorrhizal interactions can drive plant productivity, diversity, and ecosystem function; however, gaps remain in our understanding of interactions among plants and different mycorrhizal types. Species in the *Pinaceae* primarily associate with ectomycorrhizal (ECM) fungi; however, some species can be colonized by arbuscular mycorrhizal (AM) fungi as rudimentary hosts. Interactions between plants and mycorrhizal fungi are expected to change along the mutualism-parasitism spectrum with changes in resource availability; however, the impacts of AM fungi on rudimentary hosts under different resource conditions are unknown.

## METHODS

In this study, we assessed the individual and interactive effects of ECM and AM inoculation on *Pinus strobus* seedlings under factorial combinations of low, medium, or high nitrogen (N) and phosphorus (P) supply.

## RESULTS

No AM colonization was observed at the end of the experiment; however, seedlings inoculated with AM fungi showed significantly reduced growth. AM inoculation also had the greatest effect on *P. strobus* tissue nutrient concentrations under low N fertilization including increased P and reduced Mn and N:P ratio. ECM colonization benefited *P. strobus* growth and P uptake, especially under low P availability, but ECM colonization rate itself was limited by low P. ECM colonization also improved seedling regulation of N:P ratio near optimum compared to uncolonized seedlings.

## CONCLUSION

We conclude that the effects of ECM and AM inoculation on *P. strobus* are context dependent, which may have important implications for regeneration and early growth of *P. strobus* in natural and managed forests.

## Introduction

Interactions between plants and their fungal associates play an important role in plant performance with significant implications for agriculture and forestry. The vast majority of plants associate with mycorrhizal fungi, which are obligate symbionts that typically improve plant access to soil resources in exchange for plant assimilated carbon (Smith and Read 2008). Most plant species primarily associate with one type of mycorrhizae, often one of the two dominant types; arbuscular mycorrhizal (AM) fungi

which associate with ~ 80% of land plants, and ectomycorrhizal (ECM) fungi which associate with many tree species of temperate and boreal regions (Wang and Qiu 2006; Smith and Read 2008; Tedersoo and Brundrett 2017). However, plant roots are likely to come into contact with mycorrhizal fungi in soil for which they are not hosts and these interactions and their impacts on plant performance are not well understood (Giovannetti and Sbrana 1998; Dickie et al. 2001; Cosme et al. 2018).

Recent debates suggest an urgent need to accurately categorize mycorrhizal host plant types; however, mycorrhizal interactions are complex and may vary along a spectrum of host compatibility rather than representing two distinct categories, host and non-host (Brundrett and Tedersoo 2018, 2020; Sun et al. 2018, 2019; Tedersoo et al. 2019; Bueno et al. 2019, 2021). For example, members of the *Pinaceae* primarily form ECM associations; however, some species have been shown also to form rudimentary interactions with AM fungi (Cázares and Trappe 1993; Horton et al. 1998; Wagg et al. 2008; Dučić et al. 2009). These rudimentary AM (RAM) involve formation of intraradical hyphae and vesicles (fungal storage structures) but very few or no arbuscules (primary sites of resource transfer in the plant-fungal mutualism) (Cosme et al. 2018). Because of the lack of arbuscules, AM are not considered mutualistic in these cases, and negative effects of RAM on *Arabidopsis* species suggest that they are potentially parasitic (Veiga et al. 2013). Indeed, AM inoculation has been shown to reduce growth of both colonized and uncolonized eastern white pine (*Pinus strobus* L.) seedlings by over 20% (Wagg et al. 2011). In contrast, Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) seedlings showed no difference in biomass with AM colonization (Smith et al. 1998), and Arizona longleaf pine (*Pinus engelmannii* Carrière) and Gregg's pine (*Pinus greggii* Engelm.) showed positive growth responses to AM inoculation (Montes-Rivera et al. 2001; Franco-Ramírez et al. 2021). Therefore, the overall effects of AM fungal interactions with members of the *Pinaceae* are context dependent and not fully understood.

Effects of AM fungi on hosts may vary for multiple reasons, including soil resource stoichiometry (Johnson 2010; Holste et al. 2016; Jin et al. 2017). Plant response to mycorrhizal inoculation depends on the cost-benefit ratio of the exchange of carbon for nutrients. Under P limitation, AM fungi typically improve host plant fitness by improving nutrient uptake, but with increasing P fertilization plants tend to benefit less from AM fungi or show negative growth responses (Johnson et al. 1997; Hoeksema et al. 2010; Corrêa et al. 2014). Nitrogen availability interacts with P to play a role in the symbiosis as low P combined with low N can limit the mutualism, further reducing the benefit of AM fungi to plants, and high P with high N can promote parasitic interactions (Amijee et al. 1989; Johnson 2010; Nouri et al. 2014). AM fungi can also reduce host Mn concentrations (Lehmann and Rillig 2015; Lambers et al. 2021). It remains unknown whether these same principles apply for RAM host plants such as members of the *Pinaceae*.

Soil resource stoichiometry also influences interactions of ECM fungi and host plants. ECM can be essential in overcoming host plant N limitation; however, in some cases ECM fungi can drain host plant C and immobilize N (Näsholm et al. 2013; Kaiser et al. 2014; Terrer et al. 2016; Ågren et al. 2019). High N fertilization inhibits ECM fungal growth and disrupts the benefit of ECM to plants (Arnebrant 1994; Treseder 2004; Kivlin et al. 2013). Combined addition of N and P can reduce plant dependence on ECM

fungi, but low P can promote ECM formation even with high N availability (Wallander and Nylund 1992; Mayor et al. 2015). Under current accelerating global change, where anthropogenic activities are altering soil fertility and resource stoichiometry on global scales, it is essential to understand how these changes will affect plant-microbe interactions (Peñuelas et al. 2013; Lilleskov et al. 2018; Treseder et al. 2018).

*Pinus strobus* is an ecologically and economically important tree species in eastern North America. It was once a dominant forest tree, but 150 + years of heavy exploitation and emerging pests and pathogens have resulted in fragmented populations and reduced abundance (Rajora et al. 2000; Costanza et al. 2018). Still, it remains one of the highest volume sawtimber species in New England, USA (Butler et al. 2015; Morin et al. 2015). *Pinus strobus* now commonly occurs in mixed forests with AM-forming tree species (e.g., *Acer*, *Prunus*, *Fraxinus*) as well as a variety of shrubs and herbaceous plants. Inoculation with ECM fungi during nursery production of *P. strobus* improves growth and out-planting success (Castellano 1996).

In this study, we assessed how fertilization with N and P influences the interactions between *P. strobus*, AM, and ECM fungi. We asked: 1) how do AM and ECM interact with N and P supply to influence *P. strobus* seedling growth and nutrition, and 2) do mycorrhizae influence *P. strobus* seedling regulation of N and P tissue stoichiometry? We hypothesized that the effects of AM and ECM inoculation of *P. strobus* depend on soil resource supply and N:P stoichiometry.

## Methods

### *Experiment 1*

#### *Seedling, potting medium, and mycorrhizal inoculum preparation*

*Pinus strobus* seeds (Ontario Seed Company, Kitchener, ON, Canada) were surface sterilized with 10% bleach solution, rinsed with DI water, and then soaked in water for 24 hours. The seeds were drained and transferred to moistened paper towels and refrigerated at 3°C for 60 days. Seeds were then planted into germination trays with autoclave sterilized sand. They were grown in these trays until true needles were starting to form.

The potting medium consisted of 1:1:1 mix of sand, Turface (PROFILE Products LLC, Buffalo Grove, IL), and local topsoil from the Ontario Forest Research Institute arboretum in Sault Ste. Marie, Ontario. The arboretum soil is silty loam with a pH of 5.7, 0.13% total N and 1.81% total C. Extractable P (Bray and Kurtz 1945), total Ca, K, and Mg contents were 8.0, 179.0, 26.6, and 28.5 mg kg<sup>-1</sup> dry soil, respectively (Koyama et al. 2017). The potting medium was autoclave sterilized for 1.5 hours at 121°C twice on consecutive days. Two kg of sterile soil mixture was added to each 10 cm square by 36 cm tall (2.83L) Treepot (Stuewe & Sons, Inc.).

AM inoculum was acquired from the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM; West Virginia University) and consisted of a mix of AM taxa

including *Rhizophagus intraradices* (FL740), *Rhizophagus clarus* (GA980), *Claroideoglossum etunicatum* (UT315A), *Septoglomus deserticola* (CA113), *Acaulospora colombiana* (CL356), *Gigaspora margarita* (NC121A) in a 2:2:2:2:1:1 mix.

ECM inoculum was prepared using the commercial product Root Rescue Transplanter MS-CS™ (Root Rescue Environmental Products Inc., Waterdown, ON, Canada). Root Rescue contains both AM and ECM spores; therefore, to isolate ECM spores, 45 g of the inoculum was mixed with 2L DI water and filtered twice through a 20 µm filter to remove the AM fungal spores (AM fungal minimum spore size is typically >40 µm (Aguilar-Trigueros et al. 2019), whereas ECM spore size is typically <15 µm (Bässler et al. 2015)). The ECM fungal species in the inoculum included species known to associate with pines: *Rhizopogon villosullus*, *R. luteolus*, *R. amylopogon*, *R. fulvigleba*, *Pisolithus tinctorius*, *Laccaria bicolor*, *L. laccata*, *Suillus granulatus*, and *S. punctipes*.

In November 2018, individual *P. strobus* seedlings were transplanted into pots which were fully randomized in a greenhouse at the Ontario Forest Research Institute with mean daytime and nighttime temperatures of 25.8 and 18.3°C, respectively. Natural lighting was supplemented with high pressure, sodium vapor lamps suspended above the benches with a 14-hour photoperiod. Seedlings were randomly assigned one of the four mycorrhizal inoculum treatments (none, AM, ECM, AM+ECM) and one of nine nutrient regimes with eight replicates for a total of 288 pots. To control for the abiotic effects of the inoculum, sterile AM and ECM inoculum was created by autoclaving a portion of the inoculum for 20 and 30 min, respectively. Mycorrhizal treatments included additions of 5 g of either live or sterile AM inoculum and 2 ml of either live or sterile ECM inoculum to the roots while planting *P. strobus* seedlings.

Fertilization treatments consisted of a factorial combination of low, medium, or high N and low, medium, or high P fertilization levels. The high fertilization treatment was based on a modified Hoagland's solution, the medium treatment reduced the high N and P levels by a factor of three, and the low fertilization treatment reduced N and P by a factor of 20 (see Table 1 for N and P concentrations in each treatment) (Hoagland and Arnon 1950; Landis et al. 1989). These levels were derived from previous studies of growth responses of pine seedlings and interaction with ECM fungi (Ingestad 1962; van den Driessche and Wareing 1966; Dumbroff 1968; Reid et al. 1983; Elliott and White 1994; Green et al. 1994). Nitrogen was added as a 1:1 ratio of  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  and  $\text{NH}_4\text{NO}_3$ , and P as  $\text{KH}_2\text{PO}_4$ . Concentrations of K, Ca, Mg, Fe, B, Mn, Zn, Cu, and Mo were kept constant across the treatments (K- 197.5, Ca- 150.4, Mg-24.3, Fe-11.2, Bo-0.27, Mn-0.11, Zn-0.13, Mo-0.05, Cu-0.03 ppm). S (107-179.9 ppm) and Cl (1.8-101.5 ppm) salts were used to adjust the concentrations of N and P and these were kept within a reasonable range (Handreck 1986; Browder et al. 2005; Kafkafi 2013). All fertilizer treatments were adjusted to pH 5.5 with HCl.

Seedlings were watered twice per week and fertilized weekly. Pots were able to drain freely. The first week after transplanting, seedlings were fertilized with 50 mL per pot because of their small size and lower demand for water and nutrients; the second week with 75 mL per pot, and then 100 mL per pot throughout the rest of the experiment (28 weeks and 27 fertilizations). Eight seedlings died before the

completion of the experiment, six of which were in the low P treatment, and these were excluded from analysis (final n = 280; Table S1).

In June 2019, basal diameter was measured with a digital caliper and then aboveground biomass was cut at the soil surface and dried at 60°C for at least 48 hours. Belowground biomass was removed from the soil, washed, and patted dry with paper towels. The fresh weight of the whole root system was recorded and then a subsample of roots was collected for mycorrhizal quantification. The remaining fresh root system was weighed again and then dried at 60°C for at least 48 hours. The dry weight of the root system after the subsamples were removed was then used to estimate the total dry belowground biomass.

Roots were cleared and stained for AM colonization with a modified ink and vinegar method using Sheaffer ink (Providence, RI, USA) (Vierheilig et al. 1998). Roots were then cut into ~1 cm segments, placed in tissue cassettes, cleared in 10% KOH for 10-12 hours at 90°C, rinsed with DI water, and bleached in 5% sodium hypochlorite for 2 min. Roots were then rinsed again with DI water and stained with ink and vinegar for 20 min and mounted on microscope slides. AM colonization was assessed under 40X magnification using a gridline intersect method with 100 intersections per sample (McGonigle et al. 1990). ECM colonization of root tips was quantified using a gridline intersect method under a dissecting microscope inspecting 100 root tips per sample (Brundrett et al. 1994). Where ECM colonization was observed, colonization ranged from 25 to 89%.

The oven-dried aboveground *P. strobus* tissues were ground to a fine powder in a Retsch Mixer Mill MM400 (Haan, Germany) for 1.5 min at 20 Hz. To acquire sufficient sample for the nutrient analyses for seedlings with less than the minimum required mass, samples were pooled within treatment combinations (final sample size- N: 274, P: 267, Mn: 266). A portion of the ground samples was used to measure total C and N concentrations in duplicate with a Flash 2000 elemental analyzer (Thermo Scientific, Cambridge, UK). The remaining ground samples were then digested and analyzed for P and Mn by inductively coupled plasma mass spectrometry (ICP-MS) at the Ontario Forest Research Institute.

## *Experiment 2*

A second experiment was conducted starting in October 2020 to confirm the viability of the AM inoculum and to test for a microbial filtrate effect (microbes other than AM fungi in the inoculum). To confirm the viability of the inoculum, we grew *Sorghum × drummondii* (an AM host plant) with the same live AM inoculum used in Experiment 1. To test for a microbial filtrate effect, we grew *P. strobus* with either the whole AM inoculum, a microbial filtrate from the AM inoculum, or sterilized AM inoculum with eight replicates. The microbial filtrate was created by filtering the AM inoculum through a 20 µm filter and sterilized inoculum was autoclaved for one hour. Seeds, pots, and soil medium were treated the same as Experiment #1, but *P. strobus* was grown in a walk-in growth chamber with daytime temperature- 23°C, nighttime temperature- 20°C, and 16-hour photoperiod. The plants were fertilized with the medium N, high P fertilizer described above. Plants were harvested in May 2021 in the same way as in Experiment 1.

*Sorghum* roots were cleared and stained for AM colonization in the same way as the *P. strobus* roots except that clearing time was reduced to 20 min and bleaching was not necessary.

### Statistical Analyses

To test for treatments effects on the probability of ECM colonization, we used a binomial model with a logit link including AM inoculation, N, and P fertilization as main effects and number of colonized versus uncolonized seedlings as the response variable. Additional *P. strobus* seedling data were analyzed with general linear models. Because no AM colonization was observed, AM inoculation was used as a predictor variable. Presence of ECM colonization (rather than inoculation) was used as a categorical predictor variable. Results were very similar when ECM colonization was treated as a continuous variable. Seedlings inoculated with ECM but with no observed colonization were removed for further analyses. Additional predictor variables included levels of N and P fertilization as continuous variables (ppm) and all possible 2-way interactions. Response variables included *P. strobus* seedling diameter, total biomass, root:shoot ratio, shoot N, P, and Mn concentrations, and shoot N:P ratio. Plants' capacity to regulate N and P uptake can be inferred from the slope of the regression of the log of the N:P ratio of aboveground biomass vs log of the N:P supply ratio, with the regulatory coefficient  $H$  represented by the inverse of this slope (Sterner and Elser 2002). We ran a linear model to compare the slope of N:P uptake vs N:P supply in the different mycorrhizal treatments. This model included the response of the log of seedling tissue N:P ratio to the log of the fertilizer N:P ratio and the AM and ECM treatments. All response variables except Mn concentration were Box-Cox transformed to meet the model assumptions. For Experiment 2, *P. strobus* biomass response to sterile inoculum, microbial filtrate, or live inoculum was tested with an ANOVA. All analyses were performed in R version 4.0.3 (R Core Team 2020).

## Results

### Mycorrhizal colonization

Of the *P. strobus* seedlings inoculated with ECM in Experiment 1, 14 (10%) showed evidence of ECM colonization. Of these, 71% were in the ECM only treatment and 29% in the AM + ECM treatment ( $z_{1,136} = -1.7$ ,  $p = 0.091$ ; Fig. S1A). Probability of ECM colonization was higher with increased P fertilization ( $z_{1,136} = 2.0$ ,  $p = 0.047$ ; Fig. S1B) and no seedlings were colonized by ECM in treatments with N:P ratio higher than 10. We observed no evidence of AM colonization of *P. strobus*; however, in Experiment 2, we confirmed colonization of *Sorghum × drummondii* grown with the same AM inoculum, indicating that the inoculum was viable.

### Biomass and allocation

*P. strobus* seedling total biomass and diameter increased with P fertilization (708% and 144% increase in low vs. high P, respectively). Seedling biomass was 11% lower with high N compared to the low N fertilization (Fig. 1A) and seedling basal shoot diameter did not change with N fertilization. Root:shoot ratio declined with increasing levels of both N and P (Fig. 1B, Table S2).

Seedlings inoculated with live AM fungi had 27% lower total biomass and 12% smaller diameter than seedlings with sterilized AM ( $P < 0.001$ ; Table S2). In Experiment 2, we did not observe any effect of the microbial filtrate from the AM inoculum on *P. strobus* seedling biomass (Treatment:  $F_{2,21} = 0.19$ ;  $p = 0.83$ ; Fig. S2).

*Pinus strobus* seedlings with ECM colonization had 80% higher biomass and had 36% larger diameter than seedlings without ECM colonization, and this difference was greatest under low P (311% higher biomass for ECM colonized vs uncolonized seedlings) (ECM x Phos:  $F_{1,139} = 11.7$ ;  $p = 0.001$ ; Fig. 2A). Under low P fertilization, root:shoot ratio was 31% lower for seedlings with ECM colonization than those without, and this effect diminished with increased P (ECM x Phos:  $F_{1,139} = 10.8$ ,  $p = 0.001$ ; Fig. 2B; Table S2).

## Tissue nutrient concentration

*P. strobus* shoot tissue P concentration [P] increased as P fertilization increased but decreased as N fertilization increased. Seedlings with ECM colonization had two times higher [P] than seedlings not colonized by ECM and this effect was most pronounced under low P supply (ECM x Phos:  $F_{1,132} = 23.2$ ,  $p < 0.001$ ; Fig. 3A). N fertilization modified the effect of AM inoculum on [P]; that is, AM inoculation increased [P] under low N but decreased [P] under high N (AM x Nit:  $F_{1,132} = 6.1$ ,  $p = 0.015$ ; Table S3; Fig. S3A).

Seedling shoot tissue N concentration [N] increased as N fertilization increased but decreased as P fertilization increased. Overall, [N] was 24% lower in seedlings with ECM colonization than those without ECM colonization, and this effect was most pronounced under low P (ECM x Phos:  $F_{1,135} = 4.4$ ,  $p = 0.037$ ). P fertilization also influenced the effect of AM inoculum on [N]; that is, AM had a negative effect on [N] under low and medium P fertilization (-10%) but a slight positive effect under high P (+6%) (AM x P:  $F_{1,135} = 5.2$ ,  $p = 0.024$ ; Fig. 3B; Table S3).

Overall, shoot tissue Mn concentration [Mn] decreased as P fertilization increased, but increased as N fertilization increased. At high N fertilization, [Mn] remained high and did not change with increased P fertilization (Phos x Nit:  $F_{1,132} = 7.8$ ,  $p = 0.006$ ; Table S3). Inoculation with AM fungi reduced seedling [Mn] by 26% overall compared to uninoculated seedlings (AM:  $F_{1,132} = 39.4$ ,  $p < 0.001$ ), and had a stronger negative effect on [Mn] under low N fertilization (-45% at low N, -27% at medium N, and -11% at high N) (AM x N:  $F_{1,132} = 7.1$ ,  $p = 0.009$ , Fig. S3C). ECM colonization reduced [Mn] by 10% on average and this effect varied with P fertilization (ECM x P:  $F_{1,132} = 4.4$ ,  $p = 0.039$ ; Fig. 3C).

The N:P ratio of aboveground tissues decreased as P fertilization increased and increased as N fertilization increased. Overall, seedlings colonized with ECM had 66% lower tissue N:P ratio than seedlings not colonized with ECM (ECM:  $F_{1,132} = 89.2$ ,  $p < 0.001$ ) and this difference was largest at low P

fertilization (-85%) (ECM x Phos:  $F_{1,132} = 21.3$ ,  $p < 0.001$ ; Fig. 3D). AM inoculation resulted in lower tissue N:P ratio, but only at low N fertilization (-22%) (AM x Nit:  $F_{1,132} = 6.4$ ,  $p = 0.012$ ; Table S3, Fig. S3D).

We evaluated the relationship between the N:P stoichiometry of aboveground tissue and that of the added fertilizer. The log of N:P ratio of pine aboveground biomass showed a linear relationship with the log of N:P ratio of fertilizer supplied with an average slope of 0.34. AM inoculation did not significantly affect the slope of this relationship (AM: 0.37, None: 0.32). ECM colonization altered the relationship between tissue N:P ratio and fertilizer N:P ratio by greatly reducing the slope (0.02) compared with the slope of the relationship for seedlings not colonized by ECM (N:P ratio x ECM:  $F_{1,140} = 16.2$ ,  $p < 0.001$ ; Fig. 4). Tissue N:P was also related to the N and P fertilization rates where low fertilization resulted in higher N:P ratios than high fertilization at the equivalent fertilizer N:P ratio (Fig. S4).

## Discussion

Our results indicate that *P. strobus* seedlings growth and nutrition are influenced by ECM colonization and AM inoculation and their interactions with N and P fertilization. In our experimental system, colonization by ECM promoted seedling growth by overcoming P limitation, but ECM colonization was suppressed under low P fertilization (Cairney 2011). Inoculation with AM fungi resulted in reduced seedling growth even though no colonization of roots by AM fungi was observed. Moreover, AM inoculum and ECM colonization both influenced seedling nutrition depending on resource supply. These results have important implications for understanding the interactions between ECM and AM under varying soil resource availability.

While previous studies have found RAM colonization of *P. strobus*, we observed no AM colonization (Wagg et al. 2008, 2011). It has been suggested that RAM colonization is more common in members of the *Pinaceae* when an AM host plant is present, which may explain the lack of colonization in our study where *P. strobus* was grown for over six months without an AM host (Smith et al. 1998; Wagg et al. 2011). AM spores can germinate and initiate hyphal growth without the presence of a host plant but cannot persist long-term without a host, so it is possible that RAM developed at the beginning of the experiment but was not sustained over time due to the lack of a suitable host plant (Giovannetti and Sbrana 1998).

Despite the lack of colonization, AM inoculation had negative effects on *P. strobus* performance that did not vary with resource stoichiometry. Because we found no negative effects of the microbial filtrate associated with the AM inoculum in experiment 2, we attribute these negative effects to AM fungi or microbes obligately associated with the mycorrhizosphere (Nogueira et al. 2007). Negative effects of AM fungi may occur in the absence of colonization if signaling between plant and fungus stimulates a costly defense response in the plant such as local immune response or defense priming (Van Wees et al. 2008; Veiga et al. 2013; Jin et al. 2017). However, if this is the case, AM inoculation may be beneficial to RAM hosts like *P. strobus* in the presence of enemies. This possibility deserves further investigation.

Previous studies suggest that excess N can hinder ECM associations, but that P limitation can promote ECM even under high N availability (Godbout and Fortin 1990; Wallander and Nylund 1992; Treseder 2004). In our study, no ECM colonization was observed for fertilization treatments with N:P ratios higher than 10. Therefore, P limitation did not promote ECM formation in spite of high N supply, suggesting that in some cases P starvation may limit the ability of ECM fungi to colonize *P. strobus*, perhaps because of fungal P requirements or inadequate carbon supply from the plant to ECM fungi (Zhang and Elser 2017).

The range of P availability influenced the effects of ECM colonization on *P. strobus*; however, due to the low ECM colonization rates, results should be interpreted with caution. In spite of the small sample size, we found a strong positive effect of ECM colonization on *P. strobus* performance, with the greatest benefit of ECM under low P fertilization (Bougher et al. 1990; Cairney 2011). Under optimal partitioning theory, plants allocate resources to the tissue that most limits growth; for example, under strong light limitation plants allocate more to leaves, whereas under nutrient limitation plants allocate preferentially to roots (McCarthy and Enquist 2007). For non-ECM seedlings, increased P fertilization decreased root:shoot ratio, indicating lower belowground allocation corresponding with lower nutrient deficiency (Kleczewski et al. 2011). However, for ECM colonized plants, there was little change in allocation with increasing P fertilization demonstrating that ECM may alter biomass allocation patterns by increasing nutrient access (Chen et al. 2016; Cheng et al. 2016). The root:shoot ratio also decreased with higher N fertilization, indicating that despite the lack of strong total biomass response, biomass allocation of *P. strobus* responded strongly to the N treatment.

The consistent large growth response of seedlings to P fertilization suggests that P was limiting in all treatments (Koerselman and Meuleman 1996; Güsewell 2004). In contrast, seedling growth showed small, neutral, or negative response to increasing N fertilization. The strong response of biomass allocation to N fertilization could suggest N and P co-limitation at low N supply. The shoot N:P ratios also suggest there could be co-limitation by N and P in certain treatments. Seedlings in treatments with N:P supply ratios less than 6 (high P – low N, high P – medium N, and medium P – low N) had average tissue N:P ratios between 10 and 20, the range proposed by previous studies for the switch between N and P limitation or co-limitation (Koerselman and Meuleman 1996; Güsewell 2004). In the low P treatment, average N:P ratios were 54.0 for seedlings without ECM colonization versus 8.2 for seedlings with ECM colonization. ECM colonized seedlings had greatly increased tissue [P] and reduced tissue [N], especially under low P. Furthermore, average N:P tissue ratios of ECM colonized seedlings remained close to optimal (~ 10) across all the fertilizer treatments where colonization was observed (Knecht and Göransson 2004).

Using the relationship between the N:P ratio of the fertilizer and the N:P ratio of the seedling tissues, we estimated the plants' capacity to regulate N and P uptake, the regulatory coefficient  $H$  (Sternner and Elser 2002; Riley et al. 2019). For seedlings not colonized by ECM,  $H$  was 2.95; where values of  $H$  closer to 1 indicate minimal homeostatic regulation, i.e., tissue N:P ratio is closely related to N:P supply. For seedlings colonized by ECM, the regulatory coefficient  $H$  was 47.14, indicating that ECM colonization increased homeostatic regulation in N:P uptake, which is beneficial for preventing deficiency and toxicity under variable nutrient supply rates (Güsewell 2004; Kranabetter et al. 2019; Riley et al. 2019). ECM

colonization appeared to alleviate P limitation and potentially promote N:P co-limitation across the low N:P supply treatments. Although northern forests have traditionally been considered to be most limited by N, a long history of high anthropogenic N deposition may have shifted many forests towards limitation by P, or N and P co-limitation (Elser et al. 2007; Vitousek et al. 2010; Harpole et al. 2011; Goswami et al. 2018). Thus, understanding how P limitation may influence mycorrhizae and plant growth is highly relevant in our changing global environment.

Fertilization regime did not alter the negative effect of AM inoculation on seedling biomass but did alter the effect of AM on shoot nutrient concentrations. AM inoculation increased [P] at low N but decreased [P] at high N. AM had a negative effect on tissue [N], especially under low P fertilization, thereby decreasing N uptake relative to P (Riley et al. 2019). Together the effects on [N] and [P] resulted in lower N:P ratios of seedlings inoculated with AM fungi, especially in treatments where there was potential N and P co-limitation. AM inoculation reduced seedling [Mn] and the greatest effect of AM on [Mn] was observed with low N supply. Meta-analysis has shown that AM fungi can have overall negative effects on woody host plant [Mn] especially when P is limiting (Lehmann and Rillig 2015), but our study shows that this can also be the case for a RAM host. AM fungi are also known to alter soil microbiomes in ways that alter soil Mn availability. For example, AM fungi can influence the abundance of Mn-reducing and Mn-oxidizing bacteria, thereby changing Mn availability to plants, but the direction of these changes appear to be context dependent, potentially depending on soil pH (Kothari et al. 1991; Posta et al. 1994; Nogueira et al. 2007). It is unlikely that Mn is limiting or at toxic levels under these conditions, so the connection between reduced [Mn] and reduced biomass are unclear (Kavvadias and Miller 1999; St.Clair and Lynch 2005).

ECM fungi can promote positive plant-soil feedbacks for host trees (Bennett et al. 2017; Kadowaki et al. 2018). The negative effects of AM inoculum on pines may be an overlooked mechanism promoting positive plant-soil feedbacks, where performance is improved by soil from conspecific individuals compared with soil from heterospecifics. For example, if AM inoculum in soil is increased by a heterospecific AM host species but reduced by the presence of a conspecific RAM host then conspecific soil would benefit pine performance (Kadowaki et al. 2018). Recent studies have shown that differences in mycorrhizal type have important implications for plant-soil feedbacks and population dynamics in temperate forests where pines are a major component (Bennett et al. 2017). In addition, from an applied standpoint, our findings suggest that commercial mixed AM and ECM inoculum may not be the best option for *P. strobus* seedlings, and that ECM inoculum alone should be favored. Furthermore, ECM colonization can alleviate P limitation and increase homeostatic regulation of tissue N:P ratio, but very high N:P supply can limit ECM colonization. As pines are important components of forests ecosystems and forestry species worldwide, the impacts of interactions between AM and ECM inoculum on growth and nutrition of pines is of great interest.

## Declarations

## Acknowledgements

This research was conducted in Robinson-Huron Treaty territory and the traditional territory of the Anishnaabeg, specifically the Garden River and Batchewana First Nations, as well as Métis People. We thank Rachelle Norman, Katja Karhi, Jen Bridge, Caitlyn Horsch, Aki Koyama for assistance with setting up the experiment and sample analysis, Darren Derbowka and Scott Bowman for technical assistance, and Tim Fahey for comments on the manuscript.

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### *Conflicts of interest/Competing interests*

None.

### *Availability of data and material*

Data will be made available if accepted for publication.

### *Code availability*

Not applicable.

### *Authors' contributions*

PMA, FWB, and CF designed, set up, and harvested Experiment 1; PMA and CF designed, set up, and harvested Experiment 2, CF maintained the experiments, analyzed the data, and wrote the first draft; and all authors contributed to and approved the final draft.

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## Tables

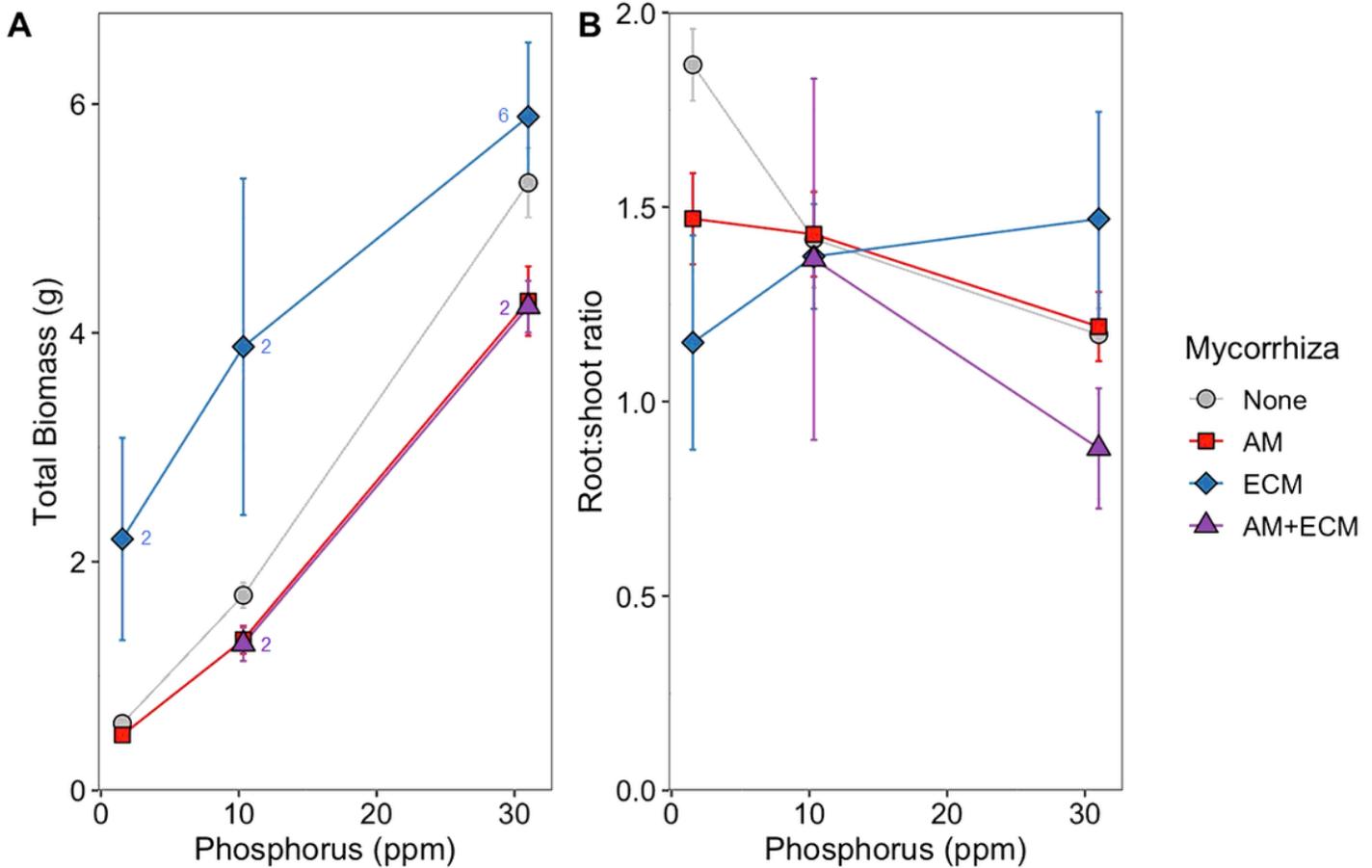
**Table 1.** N and P concentrations (ppm) and N:P ratio in the fertilizer aqueous solutions. (LN- low nitrogen, MN- medium nitrogen, HN- high nitrogen, LP- low phosphorus, MP- medium phosphorus, HP- high phosphorus).

N treatment	P treatment	N (ppm)	P (ppm)	N:P ratio
LN	LP	10.5	1.55	6.77
LN	MP	10.5	10.33	1.02
LN	HP	10.5	31	0.34
MN	LP	70	1.55	45.16
MN	MP	70	10.33	6.78
MN	HP	70	31	2.26
HN	LP	210	1.55	135.48
HN	MP	210	10.33	20.33
HN	HP	210	31	6.77

## Figures

Figure 1

*Pinus strobus* L. seedling A) total biomass and B) root:shoot ratio (mean  $\pm$  SE) response to N and P fertilization treatments (N =15-19 per treatment).

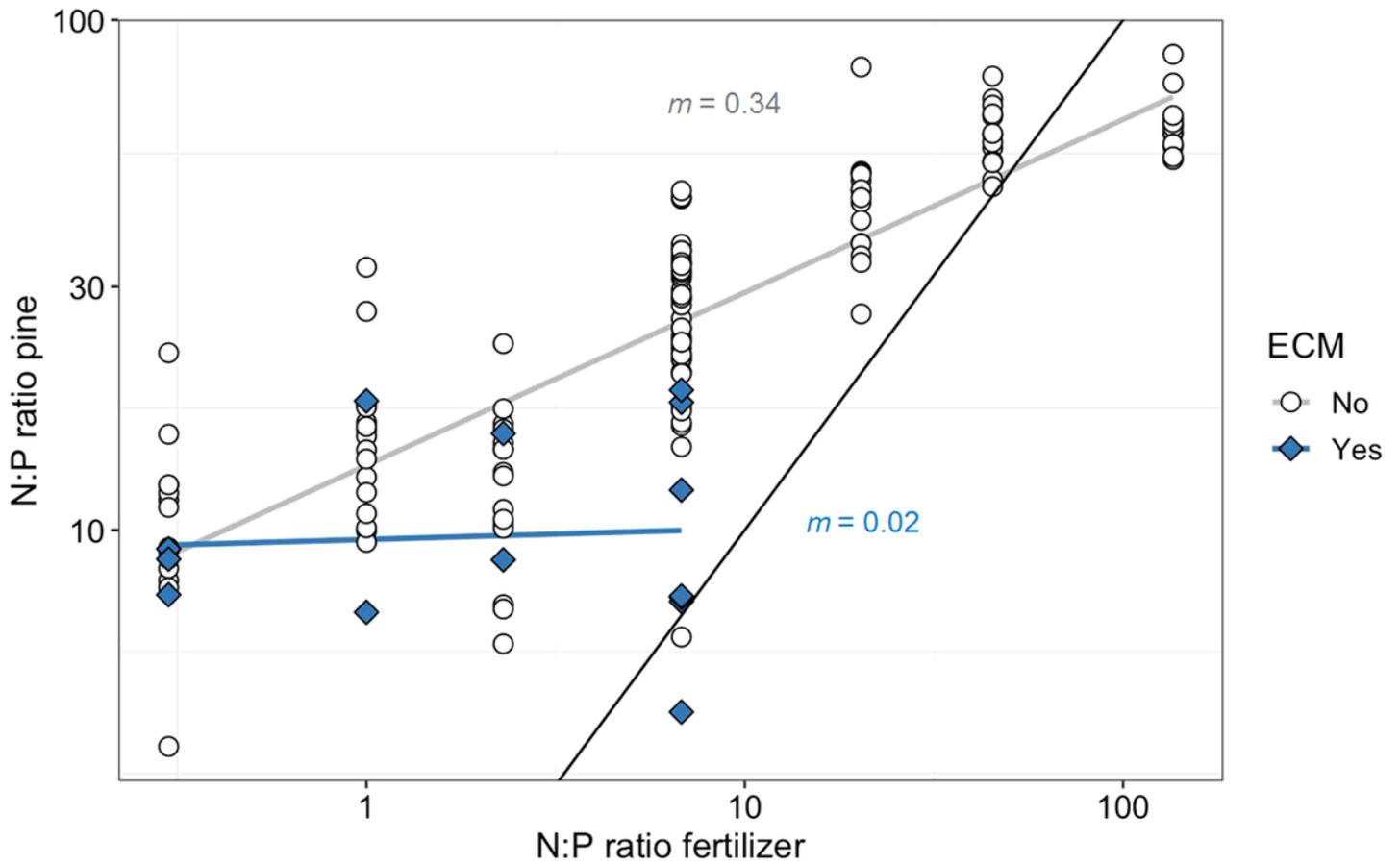


**Figure 2**

A) Total *P. strobus* L. seedling biomass and B) root to shoot ratio (mean  $\pm$  SE) response to P fertilization and mycorrhizal treatment. Ectomycorrhizal (ECM) treatment includes only seedlings with ECM colonization and arbuscular mycorrhizal (AM) treatment includes all seedlings inoculated with AM fungi. Numbers next to points for ECM and AM+ECM treatments represent N for those treatments as limited numbers of seedlings were colonized by ECM. For None and AM treatments, N = 24. Sample sizes are equal for panel A and B.

**Figure 3**

*Pinus strobus* L. seedling aboveground tissue A) P concentration, B) N concentration, C) Mn concentration, and D) N:P ratio (mean  $\pm$  SE) response to P fertilization and mycorrhizal treatment. Ectomycorrhizal (ECM) treatment includes only seedlings with ECM colonization and arbuscular mycorrhizal (AM) treatment includes all seedlings inoculated with AM fungi. Sample sizes for ECM and AM+ECM treatments are equal to those in Figure 2. For None and AM treatments N = 19-24.



**Figure 4**

Log of N:P ratio of aboveground *P. strobus* L. tissue vs N:P mass ratio of aqueous fertilizer applied for seedlings with or without ECM colonization. The black line shows a 1:1 slope indicating no regulation of N:P uptake, whereas a slope equal to 0 would indicate complete regulation of N:P uptake.

## Supplementary Files

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