**Negative effects of allelopathic plant invasion accumulate as the growth season progresses**

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**Data Availability**

All data, analysis scripts, and plot scripts are publicly available at <https://github.com/eaperkowski/2023_TT_phys> (DOI: )

**Abstract**

A key trait associated with plant invasions is the production of allelopathic compounds that disrupt the plant-fungal symbioses of neighboring plants. Yet, we know remarkably little about the temporal effects of allelopathic invaders on neighboring native species over the growing season as the tree canopy closes and soil resources deplete. Here, we collected gas exchange data at two time points reflecting an open and closed tree canopy in two native understory species (Trillium spp. and Maianthemum racemosum). Gas exchange data were collected using individuals growing in a long-term Alliaria petiolata manipulation experiment. Alliaria is a widespread allelopathic forest invader that reorganizes AM fungal communities and negatively impacts native plant population dynamics. Alliaria presence decreased net photosynthesis in Trillium spp., but only after the tree canopy closed and soil nutrient availability decreased. This pattern was associated with a reduction in apparent photosynthetic capacity that stemmed from a reduction in the maximum rate of electron transport for RuBP regeneration. Alliaria presence also decreased net photosynthesis in M. racemosum. This pattern was associated with a reduction in stomatal conductance that increased the stomatal limitation of net photosynthesis, which increased in strength after the tree canopy closed and soil resource availability decreased. In both species, the detrimental effects of Alliaria intensified as the growth season progressed. These responses may have been associated with seasonal reductions in nutrient availability that increased reliance on disrupted AM fungal partners, which likely increased photosynthetic nutrient or water limitation in Trillium spp. and M. racemosum, respectively.

**Keywords**

*Alliaria petiolata*, AM fungi, photosynthesis, plant invasion, stomatal conductance, stomatal conductance, water use efficiency

**Introduction**

Invasive plants often express unique traits that increase their likelihood of establishment in novel ecosystems. Allelopathy, a ‘novel weapon’ defined as a secondary compound produced by a plant that negatively impacts neighboring plant species and soil microbial communities (Inderjit *et al.*, 2011), has gained traction as a mechanism to explain the success of invasive plant species (Callaway & Ridenour, 2004; Callaway *et al.*, 2008). Previous work estimates that ~52% of invasive plant species can produce and release allelopathic compounds into the environment (Kalisz *et al.*, 2021), leading to negative impacts on neighboring native plant performance and soil microbial community composition (Hale & Kalisz, 2012; Bialic-Murphy *et al.*, 2020; Qu *et al.*, 2021; Roche *et al.*, 2021; Zhang *et al.*, 2021; Bialic‐Murphy *et al.*, 2021). Despite the ubiquity of allelopathy in invasive plant species, few studies have investigated the impacts of allelopathic plant invasion on the physiology of coexisting native plants (but see Brouwer et al., 2015; Hale et al., 2016), particularly across the growing season. Understanding these dynamics across the growing season is crucial for clarifying the mechanisms by which invasive species impact plant-microbe interactions and their broader effects on ecosystem function.

Photosynthesis is a process in terrestrial ecosystems that links ecosystem carbon, nutrient, and water cycles and is the product of interactions between above-ground growth conditions and soil resource availability (Hungate *et al.*, 2003; Wieder *et al.*, 2015). Plants fix carbon dioxide drawn in from the atmosphere and convert it into simple sugars using enzymes that have large nutrient and energy requirements to build and maintain (Evans & Seemann, 1989; Evans & Clarke, 2019). Above-ground growth conditions such as light availability, atmospheric CO2 concentration, vapor pressure deficit, and temperature determine leaf demand to build and maintain photosynthetic enzymes, as these conditions play a direct role in regulating photosynthetic enzyme kinetics and substrate availability (Bernacchi *et al.*, 2001; Dong *et al.*, 2017, 2020, 2022; Smith *et al.*, 2019; Paillassa *et al.*, 2020; Peng *et al.*, 2021; Westerband *et al.*, 2023). Whether the demand for building and maintaining photosynthetic enzymes gets satisfied in a given environment depends on water and nutrient uptake from the soil, and allocation of these resources to photosynthetic enzymes. For example, increasing light availability increases the demand to build and maintain photosynthetic enzymes to support the increased photosynthetic capacity needed to maximize the use of available light (Walters, 2005; Smith *et al.*, 2019). This heightened demand can be satisfied in systems where soil resource availability exceeds demand, allowing plants to increase nutrient and water uptake, allocate these resources toward photosynthetic enzymes, and increase stomatal conductance. However, plants may not be able to increase photosynthetic capacity and carbon assimilation to the same extent when soil resource availability is not sufficient for satisfying heightened demand to build and maintain photosynthetic enzymes (Waring *et al.*, 2023). In such scenarios, plants may invest more heavily in resource acquisition strategies (e.g., symbioses with mycorrhizal fungi) that maximize resource uptake efficiency and increase the likelihood that demand to build and maintain photosynthetic enzymes is satisfied with minimal carbon expenditure (Bloom *et al.*, 1985; Rastetter *et al.*, 2001).

Allelopathic compounds with antimicrobial properties can facilitate reductions in plant-available nutrient availability by inhibiting the growth and reproduction of soil microbial communities that are essential for regulating ecosystem biogeochemical cycles, such as mycorrhizal fungi (Hale & Kalisz, 2012). Arbuscular mycorrhizal (AM) fungi form obligate symbioses with plants and exchange mineral nutrients and water mined from soil solution to plants in exchange for photosynthate allocated below ground (Smith & Read, 2008). Previous work indicates that antimicrobial allelopathic compounds can inhibit AM fungal spore germination, root colonization, and arbuscule formation in neighboring plants that associate with AM fungi (Cantor *et al.*, 2011), altering AM fungal community composition (Callaway *et al.*, 2008) and potentially disrupting the strength of neighboring plant-AM fungal symbioses (Johnson *et al.*, 1997; Callaway *et al.*, 2008). As a result, allelopathy can reduce plant-perceived nutrient and water availability, forcing plants to decrease nutrient and water provisioning toward structures that support leaf-level photosynthesis, reducing photosynthetic capacity, carbon assimilation, or stomatal conductance. Allelopathy may be a particularly effective invasion tactic in systems where a non-mycorrhizal species invades an AM-dominated ecosystem, ensuring that the non-mycorrhizal invading species maintains a hyper-competitive advantage over neighboring AM native species by negatively impacting AM fungal communities while not altering the strength of its own resource acquisition strategy (Roche *et al.*, 2021).

*Alliaria petiolata* (M. Bieb) Cavara & Grande (referenced herein as *Alliaria*) has become a model species for understanding the impacts of allelopathic plant invasion on soil microbial and native plant community dynamics. *Alliaria* is a brassicaceous, invasive, non-mycorrhizal, biennial herb from Eurasia that aggressively invades the understories of temperate forests in North America, releasing glucosinolates into soil environments through root exudation and leaf litter decomposition (Rodgers *et al.*, 2008). Glucosinolates are a class of antimicrobial allelochemicals that form derivative cyanide compounds that are detrimental to soil microbial and mesofaunal communities (Siebers *et al.*, 2018; Eugui *et al.*, 2022). Specifically, glucosinolates hydrolyze into allyl isothiocyanate, which inhibits AM spore germination, spore viability, root colonization, and arbuscule formation in neighboring plants (Callaway *et al.*, 2008; Cantor *et al.*, 2011). Deleterious effects of *Alliaria* presence on below-ground soil microbial community composition may scale to impact native species’ community composition, as native species that associate with AM fungi tend to be more strongly affected by *Alliaria* invasion than their non-mycorrhizal counterparts (Callaway *et al.*, 2008; Roche *et al.*, 2021).

Recent work indicates that disruptions in AM fungal community composition due to *Alliaria* invasion are associated with changes in native plant nutrient and water economics (Bialic‐Murphy *et al.*, 2021), plant population dynamics (Bialic-Murphy *et al.*, 2020), and community composition (Roche *et al.*, 2021, 2023). Changes in native plant economics due to Alliaria invasion may be associated with reduced leaf-level carbon assimilation and stomatal conductance (Hale *et al.*, 2011, 2016; Brouwer *et al.*, 2015; Bialic‐Murphy *et al.*, 2021). While native species’ photosynthetic and stomatal conductance responses to *Alliaria* invasion are directionally consistent with expectations, no studies have considered impacts of *Alliaria* invasion on indices of photosynthetic capacity, which limits our ability to understand whether these patterns are driven by water or nutrient partitioning to photosynthetic enzymes. Furthermore, photosynthetic responses to *Alliaria* presence have only been quantified later in the growth season after tree canopy closure and soil resource depletion have occurred, which limits our understanding of these patterns to a case scenario where the demand for building and maintaining photosynthetic enzymes and soil resource availability are both reduced compared to the beginning of the growth season. Studies that investigate the mechanisms that explain leaf-level photosynthetic responses to *Alliaria* at different time points in the growth session (e.g., before and after tree canopy closure) would be valuable for assessing how leaf-level physiological responses to allelopathic plant invasion compare to finer-scale impacts to AM fungal community composition. Such studies would also be valuable for detecting whether effects of allelopathic invasion on belowground fungal community dynamics scale to alter the productivity, survivorship, and composition of coexisting native populations and communities.

To determine the mechanisms driving the effects of *Alliaria* on native species physiology, we measured the gas exchange of two native understory species before and after tree canopy closure. At each timepoint, we quantified (1) net photosynthesis, (2) apparent photosynthetic capacity as the maximum rate of Rubisco carboxylation (*V*cmax) and the maximum rate of electron transport for RuBP regeneration (*J*max), which can each be limited by nutrient uptake and allocation, (3) relative chlorophyll content, which estimates nutrient allocation to light-harvesting proteins, and (4) stomatal conductance, which regulates both water transport and CO2 drawdown needed to drive photosynthetic reactions forward. We used these data to address the following hypotheses:

1. *Alliaria* presence will decrease net photosynthesis in both native species. This response will be due to a reduction in apparent photosynthetic capacity and relative chlorophyll content (indicating a reduction in nutrient provisioning to photosynthesis), a reduction in stomatal conductance (indicating a reduction in water provisioning to photosynthesis), or a reduction in both photosynthetic capacity, relative chlorophyll content, and stomatal conductance (indicating a reduction in nutrient and water provisioning to photosynthesis)
2. Negative effects of *Alliaria* presence on the leaf-level photosynthetic processes of both native species will depend on measurement time point
   1. *Alliaria* presence will negatively influence native species’ photosynthetic traits more strongly early in the growth season when the tree canopy is open, as demand for soil resources to support photosynthetic capacity will be the greatest. Increased demand should increase reliance on disrupted AM fungal symbionts to supply nutrients and water to photosynthetic leaf tissue that is needed to satisfy demand and optimize investment in photosynthetic enzymes.
   2. Alternatively, the negative effect of *Alliaria* presence on native species’ physiology will become more pronounced over the growing season, as soil resources are depleted and plants become more reliant on disrupted mycorrhizal partners for nutrient and water uptake

**Methods**

*Study site and experimental design*

This study was conducted at a long-term *Alliaria* removal experiment located at Trillium Trail Nature Reserve in Fox Chapel, PA (40.520 °N, -79.901 °W). The mean annual precipitation and mean annual temperature of the study area were 1006 mm yr-1 and 11°C, respectively (2006-2020 U.S. Climate Normals; Palecki et al., 2021). Wire fences (2.5 m tall) were set up in 2002 at five 14 x 14 m experimental plots to exclude deer and other macroherbivores. *Alliaria* was manually weeded from one half of each experimental plot since 2006, with the other half of each plot remaining unweeded. Manual weeding has been an effective strategy for *Alliaria* removal (Roche *et al.*, 2021). This long-term split-plot experiment was located on 25-75% grade slopes and soils classified as Gilpin-Upshur-Atkins soils with dominant shale, sandstone, and red clay shale bedrock components. *Alliaria* treatments were set up parallel to the slope to prevent the leaching of allelochemicals into the weeded side of the plot. A 0.5 m buffer has also been maintained between *Alliaria* treatments to minimize allelochemical leaching. Previous work conducted in this experiment has shown that *Alliaria* presence has reduced AM fungal biomass, increased AM fungal richness, and has altered AM fungal community composition (Roche *et al.*, 2021; Bialic‐Murphy *et al.*, 2021)

*Gas exchange measurements and calculations*

Between April and June 2023, gas exchange data were collected from fully expanded leaves of two perennial understory native species: *Trillium* spp. (*Trillium* *grandiflorum* (Michx.) Salisb

and *Trillium erectum* L.) and *M. racemosum*. We use *Trillium* spp. because the two species are not discernable in individuals that have not formed reproductive structures. Both species are understory perennial herbs with widespread distributions in temperate forests of North America (USDA NRCS, 2022), form rhizomes, and associate with AM fungi (Burke, 2008). Previous work notes that the timing of aboveground phenology differs between the two species: *Trillium* spp. typically emerge in April and senesce in July, while *M. racemosum* typically emerge in May and senesce as late as November (Heberling *et al.*, 2019).

Gas exchange data were collected during two measurement periods at three of the five experimental plots: once when the tree canopy was open and establishing (April 19 through April 21 for *Trillium* spp. and May 5 through May 6 for *M. racemosum*) and once when the tree canopy was fully established (June 12 through June 15 for both species). The first measurement period was conducted at separate time points for *Trillium* spp. and *M. racemosum* because of differences in the timing of full leaf expansion between species.

Net photosynthesis (*A*net; μmol m-2 s-1), stomatal conductance (*g*sw; mol m-2 s-1), and intercellular CO2 (*C*i; μmol mol-1) concentrations were measured across a range of atmospheric CO2 concentrations (i.e., an *A*net/*C*i curve) using the Dynamic Assimilation™ Technique (Saathoff & Welles, 2021), which corresponds well with traditional steady-state methods (Tejera-Nieves *et al.*, 2024). We generated all *A*net/*C*i curves along a reference CO2 ramp down from 420 µmol mol-1 CO2 to 20 µmol mol-1 CO2, followed by a ramp up from 420 µmol mol-1 CO2 to 1620 µmol mol-1 CO2 after a 90-second wait period at 420 µmol mol-1 CO2. The ramp rate for each curve was set to 200 μmol mol-1 min-1, logging every five seconds, which generated 96 data points per response curve. All *A*net/*C*i curves were generated after *A*net and *g*sw stabilized in a LI-6800 cuvette set to a 500 mol s-1 flow rate, 10000 rpm mixing fan speed, 1.5 kPa vapor pressure deficit, 25°C leaf temperature, 2000 μmol m-2 s-1 incoming light radiation, and initial reference CO2 set to 420 µmol mol-1. We extracted snapshot *A*net and *g*sw measurements using the initial measurement of each *A*net/*C*i curve at 420 µmol mol-1 CO2.

*A/Ci curve fitting and parameter estimation*

We fit *A*net/*C*i curves using the ‘fitaci’ function in the ‘plantecophys’ R package (Duursma, 2015). This function estimates the maximum rate of Rubisco carboxylation (*V*cmax; µmol m-2 s-1) and maximum rate of electron transport for RuBP regeneration (*J*max; µmol m-2 s-1) using the Farquhar et al. (1980) biochemical model of C3 photosynthesis. Triose phosphate utilization (TPU) limitation was included as an additional rate-limiting step in all curve fits. Dark respiration was estimated in curve fits as a fixed proportion of *V*cmax. Michaelis-Menten coefficients for Rubisco affinity to CO2 (*K*c; μmol mol-1) and O2 (*K*o; mmol mol-1), and the CO2 compensation point *(Γ*\*; μmol mol-1) were calculated using leaf temperature and equations derived in Bernacchi et al. (2001):

(1)

(2)

(3)

In all three equations, *T*k is the mean leaf temperature (in Kelvin) during each *A*net/*C*i curve, and R is the universal gas constant (8.314 J mol-1 K-1). All curves were visually inspected for goodness-of-fit before extracting *V*cmax and *J*max estimates.

For all *A*net/*C*i curve fits, *V*cmax and *J*max were standardized to25°C using a modified Arrhenius equation as explained in Kattge & Knorr (2007):

(4)

*k*25 represents the standardized *V*cmax or *J*max rate at 25°C, *k*obs represents the *V*cmax or *J*max estimate at the average leaf temperature measured inside the cuvette during the *A*net/*C*i curve. *H*a is the activation energy of *V*cmax (71,513 J mol-1; Kattge & Knorr, 2007) or *J*max (49,884 J mol-1; Kattge & Knorr, 2007). *H*d represents the deactivation energy of both *V*cmax and *J*max (200,000 J mol-1; Medlyn et al., 2002), and R represents the universal gas constant (8.314 J mol-1 K-1). *T*ref represents the standardized temperature of 298.15 K, and *T*obs represents the mean leaf temperature (K) during each *A*net/*C*i curve. ΔS is an entropy term that Kattge & Knorr (2007) derived as a linear relationship with the average growing season temperature of the 30 days preceding each *A*/*C*i curve (*T*g30; °C), where:

(5)

and:

(6)

We used *V*cmax25 and *J*max25 estimates to calculate the ratio of *J*max25 to *V*cmax25 (*J*max25:*V*cmax25; unitless).

*Stomatal limitation*

The extent by which stomatal conductance limited net photosynthesis (unitless) was calculated following the approach described in Farquhar & Sharkey (1982), where:

(7)

where *A*net represents the measured net photosynthesis rate where atmospheric CO2 is 420 μmol mol-1. *A*mod represents the assumed photosynthetic rate where *C*i = *C*a = 420 μmol mol-1 (that is, no stomatal resistance to gas exchange), calculated as:

(8)

*Γ*\* (μmol mol-1) is the CO2 compensation point in the absence of dark respiration, *K*m is the Michaelis-Menten coefficient for Rubisco-limited photosynthesis (μmol mol-1), and *R*d is the dark respiration rate. *K*m was calculated as:

(9)

*K*c and *K*o were calculated following Eqns. 1 and 2, while *O*i refers to leaf intercellular O2 concentration, set to 210 μmol mol-1. *R*d in Eqn. 5 was estimated as a fixed proportion of *V*cmax.

*Chlorophyll fluorescence measurements*

Relative chlorophyll content was measured after each *A*net/*C*i curve using a Soil Plant Analysis Development chlorophyll meter (SPAD, unitless) built into the MultispeQ V2.0 handheld device (PhotosynQ Inc., East Lansing, MI, USA).

*Soil characteristics*

To characterize plant-available nitrogen and phosphorus availability at the time of leaf gas exchange measurements, resin strips (Membranes International, Ringwood, NJ, USA) were placed approximately 10 cm below the soil surface to quantify mobile ammonium (ppm), nitrate (ppm), phosphate (ppm) concentrations in each plot. An initial batch of resin strips was placed in the field between April 19 and June 1, 2023, followed by a second batch inserted in the same plot location between May 30 and June 29, 2023. A total of 36 strips, 12 for each nutrient, were placed in each plot to account for the high degree of spatial heterogeneity of soil nutrient availability in temperate forests (Akana *et al.*, 2023). Cation and anion concentrations were extracted from resin strips in 0.5 M potassium sulfate at a 1:5 dilution factor. Ammonium, nitrate, and phosphate concentrations were determined through end products of colorimetric reactions (Weatherburn, 1967; Lajtha *et al.*, 1999; D’Angelo *et al.*, 2001; Doane & Horwáth, 2003). Soil inorganic nitrogen availability was estimated as the sum of the ammonium and nitrate concentrations. The soil inorganic nitrogen: phosphorus ratio was estimated as the ratio of soil inorganic nitrogen availability to soil phosphate availability.

*Data analysis*

We built a series of linear mixed-effects models to explore the role of *Alliaria* presence and measurement period on soil resource availability. Each model included *Alliaria* treatment and measurement period as categorical fixed effects, as well as an interaction term between *Alliaria* treatment and measurement period. Plot was included in the models as a random intercept term. We constructed separate models with this independent variable structure for soil nitrate availability, soil ammonium availability, soil inorganic nitrogen (nitrate + ammonium) availability, soil phosphate availability, and the soil nitrogen:phosphorus ratio. Models for soil ammonium availability, soil inorganic nitrogen availability, and the soil nitrogen:phosphorus ratio were fitted using dependent variables that were natural-log transformed to satisfy residual normality assumptions (Shapiro-Wilk: *p*>0.05).

We also built a series of species-specific linear mixed-effects models to explore the role of *Alliaria* treatment and measurement period on leaf physiological traits in *Trillium* spp. and *M. racemosum*. Species were not concatenated into a single linear mixed effect model for each trait because we did not seek to understand interspecies variability in measured traits. All models included *Alliaria* treatment (presence, absence) and measurement period (first, second) as categorical fixed effects, as well as the interaction between *Alliaria* treatment and measurement period. Plot was included in each model as a random intercept term. We constructed separate models with this independent variable structure for each species for the following dependent variables: *A*net, *g*sw, stomatal limitation, *V*cmax25, *J*max25, *J*max25:*V*cmax25, SPAD, *i*WUE, and *V*cmax25:*g*sw. In *Trillium* spp., models for *A*net, *g*sw, stomatal limitation, *V*cmax25, *J*max25, and *J*max25:*V*cmax25 were each fitted using dependent variables that were natural-log transformed to satisfy residual normality assumptions (Shapiro-Wilk: *p*>0.05). In *M. racemosum*, models for stomatal limitation, *V*cmax25, *J*max25, and *J*max25:*V*cmax25 were each fitted using dependent variables that were natural-log transformed to satisfy residual normality assumptions (Shapiro-Wilk: *p*>0.05).

Each model was fitted using the ‘lmer’ function in the ‘lme4’ R package (Bates *et al.*, 2015). Type II Wald’s χ2 and the significance (*α*=0.05) of each fixed effect coefficient was calculated using the ‘Anova’ function in the ‘car’ R package (Fox & Weisberg, 2019). We used the ‘emmeans’ R package (Lenth, 2019) to conduct post hoc comparisons using Tukey’s tests, where degrees of freedom were approximated using the Kenward-Roger approach (Kenward & Roger, 1997). All analyses and plots were conducted in R version 4.1.0 (R Core Team, 2021).

**Results**

*Soil nutrient availability*

Soil inorganic nitrogen availability was reduced by 76% and soil phosphate availability by 26% after the tree canopy closed (*p*<0.001 in all cases; Table S1; Fig. 1a-b), leading to 64% decrease in the soil nitrogen: phosphorus ratio (*p*<0.001; Table S1; Fig. 1c). Soil nitrate availability decreased by 71% after the tree canopy closed (*p*<0.001; Table S1; Fig. S1), whereas soil ammonium availability was not modified by canopy status (*p*>0.05; Table S1; Fig. S1).

*Alliaria* presence had no effect on soil inorganic nitrogen availability, soil phosphate availability, or soil nitrate availability (*p*>0.05 in all cases; Table S1; Fig. 1a-b; Fig. S1a). *Alliaria* presence marginally decreased soil ammonium availability after the tree canopy closed, but there was no effect of *Alliaria* presence prior to tree canopy closure (*Alliaria* treatment-by-canopy status interaction: *p*<0.05; Table S1; Fig. S1b). *Alliaria* presence marginally increased the soil nitrogen: phosphorus ratio (*p*=0.051; Table S1; Fig. 1c). This pattern was driven by an insignificant but positive-trending effect of *Alliaria* presence on soil inorganic nitrogen availability and an insignificant but negative-trending effect of *Alliaria* presence on soil phosphate availability (*p*>0.05 in both cases; Table S1; Fig. 1b).

**Figure 1**

**A diagram of different types of soil

Description automatically generated**

**Figure 1** Effects of tree canopy and *Alliaria* treatment on soil inorganic nitrogen availability (a), soil phosphate availability (b), and the soil N:P ratio (c). Tree canopy status is on the x-axis. Yellow points and boxplots indicate measurements collected in plots where *Alliaria* was weeded and orange points and boxplots indicate measurements collected in plots where *Alliaria* abundance was not manipulated. Boxes represent the upper (75% percentile) and lower (25% percentile) quartiles, and whiskers represent 1.5 times the upper and lower quartile values. Lettering above each treatment group indicates statistically different groups where Tukey: *p*<0.05.

*Gas exchange*

*Trillium* spp. net photosynthesis, stomatal conductance, and stomatal limitation decreased by 64%, 21%, and 58%, respectively, after the tree canopy closed (*p*<0.001 in all cases; Table 1). *Alliaria* presence decreased net photosynthesis in *Trillium* spp., but this pattern was only observed after the tree canopy closed (*Alliaria* treatment-by-canopy status interaction: *p*<0.01; Table 1; Fig. 2a). There was no effect of *Alliaria* presence on stomatal conductance or stomatal limitation in *Trillium* spp. (*p*>0.05 in both cases; Table 1) regardless of canopy status (*Alliaria* treatment-by-canopy status interaction: *p*>0.05; Table 1; Fig. 2b-c).

Net photosynthesis and stomatal conductance were reduced in *M. racemosum* by 58% and 61%, respectively, after the tree canopy closed (*p*<0.001 in both cases; Table 1). Stomatal limitation increased by 10% after the tree canopy closed (*p*<0.05; Table 1). Unlike *Trillium* spp., *Alliaria* presence decreased net photosynthesis and stomatal conductance in *M*. *racemosum* by 17% and 27%, respectively, and increased stomatal limitation by 28% (*p*<0.05 in all cases; Table 1; Fig. 2d-f). Stomatal limitation responses to *Alliaria* presence in *M. racemosum* were only observed after the tree canopy closed (*Alliaria* treatment-by-canopy status interaction: *p*<0.05; Table 1).

*Relative chlorophyll content*

SPAD values were 26% greater in *Trillium* spp. and 51% greater in *M*. *racemosum* after tree canopy closure (*p*<0.001 in both cases; Table 1). There was no effect of *Alliaria* presence on SPAD in either species (*p*>0.05; Table 1).

**Table 1** Analysis of variance results exploring the role of *Alliaria* treatment and measurement period on leaf gas exchange\*

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | ***A*net** | | ***g*sw** | | **Stomatal**  **limitation** | | ***SPAD*** | |
|  |  | df | *χ*2 | *p* | *χ*2 | *p* | *χ*2 | *p* | *χ*2 | *p* |
| *Trillium* spp. | |  |  |  |  |  |  |  |  |  |
|  | *Alliaria* presence (A) | 1 | 2.556 | 0.110 | 0.239 | 0.625 | 0.449 | 0.503 | 0.401 | 0.526 |
|  | Canopy status (C) | 1 | 1153.427 | **<0.001** | 22.758 | **<0.001** | 316.544 | **<0.001** | 77.335 | **<0.001** |
|  | A\*C | 1 | 7.693 | **0.006** | 0.413 | 0.520 | 0.404 | 0.525 | 0.653 | 0.419 |
| *M. racemosum* | |  |  |  |  |  |  |  |  |  |
|  | *Alliaria* presence (A) | 1 | 16.992 | **<0.001** | 21.318 | **<0.001** | 13.333 | **<0.001** | 2.258 | 0.133 |
|  | Canopy status (C) | 1 | 302.262 | **<0.001** | 171.471 | **<0.001** | 4.703 | **0.030** | 372.98 | **<0.001** |
|  | A\*C | 1 | 0.058 | 0.810 | 0.062 | 0.803 | 4.521 | **0.033** | 1.751 | 0.186 |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold. Key: df = degrees of freedom, *A*net = light-saturated net photosynthesis rate (μmol m-2 s-1), *g*sw = stomatal conductance (mol m-2 s-1), SPAD = relative chlorophyll content (unitless)

**Figure 2**

**A collage of different trees

Description automatically generated**

**Figure 2** Effects of *Alliaria* treatment and tree canopy status on net photosynthesis (a-b), stomatal conductance (c-d), and stomatal limitation of net photosynthesis (e-f). The left column visualizes these patterns in *Trillium* spp., while the right column visualizes these patterns in *M. racemosum*. Tree canopy status is on the x-axis. Yellow points and boxplots indicate measurements collected in plots where *Alliaria* was weeded and orange points and boxplots indicate measurements collected in plots where *Alliaria* abundance was not manipulated. Boxes represent the upper (75% percentile) and lower (25% percentile) quartiles, and whiskers represent 1.5 times the upper and lower quartile values. Lettering above each treatment group indicates statistically different groups where Tukey: *p*<0.05.

*Photosynthetic capacity*

In *Trillium* spp., *V*cmax25, *J*max25, and *J*max25:*V*cmax25 decreased by 76% and 75%, respectively, after tree canopy closure, resulting in a 5% increase in *J*max25:*V*cmax25 (*p*<0.001 in all cases; Table 2; Fig. 3a, 3c, 3e). *Alliaria* presence had no effect on *V*cmax25 (*p*>0.05; Table 2) regardless of canopy status (*Alliaria* treatment-by-canopy status interaction: *p*>0.05; Table 2; Fig. 3a). On the other hand, *Alliaria* presence decreased *J*max25 by 8% (*p*<0.05; Table 2), a pattern that was strongest after tree canopy closure (*Alliaria* treatment-by-canopy status interaction: *p*<0.05; Table 2; Fig. 3c). *Alliaria* presence had no effect on *J*max25:*V*cmax25 (*p*>0.05; Table 2; Fig. 3e).

In *M. racemosum*, tree canopy closure reduced *V*cmax25 and *J*max25 by 55% and 57%, respectively, constituting a 4% decrease in *J*max25:*V*cmax25 (*p*<0.05 in all cases; Table 2; Fig. 3b, 3d, 3f). The reduction in *J*max25:*V*cmax25 due to canopy closure was only observed in the weeded treatment (*Alliaria* presence-by-canopy status interaction: *p*<0.05; Table 2). *Alliaria* presence had no effect on *M. racemosum* *V*cmax25, *J*max25, or *J*max25:*V*cmax25 (*p*>0.05 in both cases; Table 2).

**Table 3** Analysis of variance results exploring the role of *Alliaria* treatment and measurement period on photosynthetic capacity\*

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | *V*cmax25 | | *J*max25 | | *J*max25:*V*cmax25 | |
|  |  | df | *χ*2 | *p* | *χ*2 | *p* | *χ*2 | *p* |
| *Trillium* spp. | |  |  |  |  |  |  |  |
|  | *Alliaria* presence (A) | 1 | 1.429 | 0.232 | 4.222 | **0.040** | 1.314 | 0.252 |
|  | Canopy status (C) | 1 | 1612.528 | **<0.001** | 2048.753 | **<0.001** | 8.296 | **0.004** |
|  | A\*C | 1 | 2.359 | 0.125 | 5.440 | **0.020** | 0.153 | 0.696 |
| *M. racemosum* | |  |  |  |  |  |  |  |
|  | *Alliaria* presence (A) | 1 | 0.036 | 0.849 | 0.115 | 0.735 | 1.567 | 0.211 |
|  | Canopy status (C) | 1 | 352.379 | **<0.001** | 401.28 | **<0.001** | 5.806 | **0.016** |
|  | A\*C | 1 | 0.15 | 0.698 | 0.190 | 0.663 | 4.487 | **0.034** |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold. Key: df = degrees of freedom, *V*cmax25 = maximum rate of Rubisco carboxylation at 25°C (μmol m-2 s-1), *J*max25 = maximum rate of electron transport for RuBP regeneration at 25°C (μmol m-2 s-1), *J*max25:*V*cmax25 = ratio of *J*max25 to *V*cmax25 (unitless)

**Figure 3**

**A collage of a graph

Description automatically generated**

**Figure 3** Effects of *Alliaria* treatment and tree canopy status on the maximum rate of Rubisco carboxylation (a-b), the maximum rate of electron transport for RuBP regeneration (c-d), and the ratio of the maximum rate of Rubisco carboxylation to the maximum rate of electron transport for RuBP regeneration (e-f). The left column visualizes these patterns in *Trillium* spp., while the right column visualizes these patterns in *M. racemosum*. Tree canopy status is on the x-axis. Yellow points and boxplots indicate measurements collected in plots where *Alliaria* was weeded and orange points and boxplots indicate measurements collected in plots where *Alliaria* abundance was not manipulated. Boxes represent the upper (75% percentile) and lower (25% percentile) quartiles, and whiskers represent 1.5 times the upper and lower quartile values. Lettering above each treatment group indicates statistically different groups where Tukey: *p*<0.05.

**Discussion**

Gas exchange measurements were collected at two timepoints in two understory native AM perennial species growing in a long-term *Alliaria* manipulation experiment. These measurements were used to quantify the mechanisms that drive the effects of allelopathic plant invasion on leaf-level photosynthesis of coexisting native species and to determine whether these patterns varied throughout the growing season. *Alliaria* presence decreased net photosynthesis rates in both species, but this pattern only appeared in *Trillium* spp. after tree canopy closure. Reduced net photosynthesis rates in *Trillium* spp. were associated with a reduction in apparent photosynthetic capacity that stemmed from a reduction in the maximum rate of electron transport for RuBP regeneration and no change in the maximum rate of Rubisco carboxylation or stomatal conductance. In contrast, *Alliaria* presence had no effect on apparent photosynthetic capacity in *M. racemosum*, but decreased *M. racemosum* net photosynthesis through reduced stomatal conductance and increased stomatal limitation. These findings suggest that *Alliaria* presence may have modified nutrient uptake and provisioning to photosynthesis in *Trillium* spp. and modified water uptake and provisioning to photosynthesis in *M. racemosum*. In both species, negative responses to *Alliaria* were stronger after tree canopy closure and soil nutrient and water(?) availability was decreased. These responses suggest that effects of *Alliaria* presence on native plant physiology accumulate as the growth season progresses despite occurring through different mechanisms.

*Negative effects of Alliaria presence on net photosynthesis accumulate as the growth season progresses*

We hypothesized that negative effects of *Alliaria* would vary by measurement period through two alternative pathways. First, we hypothesized that negative effects of *Alliaria* on the leaf-level photosynthesis of native species would be strongest early in the growth season. This pattern was expected because demand for building and maintaining photosynthetic enzymes in understory plants should be greatest during time periods where the tree canopy is still establishing and light availability should be greatest. We expected that disrupted AM fungal communities due to *Alliaria* presence would have stronger negative impacts on plant nutrient uptake, as individuals might invest more strongly in disrupted AM fungal symbioses to satisfy heightened leaf demand for building and maintaining photosynthetic enzymes. However, such responses were not observed.

Null effects of *Alliaria* presence on photosynthetic traits during the first measurement period may have been due to resource optimization. Resource optimization theory predicts that, given multiple potential acquisition strategies, plants should prioritize resource uptake from the strategy that minimizes the cost and maximizes the uptake efficiency of acquiring soil resources (Rastetter *et al.*, 2001; Kummel & Salant, 2006). Disruptions in AM fungal partner strength due to *Alliaria* presence could have increased the cost of acquiring soil resources through AM fungi relative to direct uptake pathways when soil resource availability was high. If true, increased nutrient availability earlier in the growth season may have caused individuals to invest more heavily in less costly and more efficient direct uptake pathways, which may have minimized or completely offset any negative effects of *Alliaria* presence on nutrient uptake through AM symbioses. Costs of nutrient acquisition were not quantified here, though would be a useful measurement to deduce whether resource optimization was the mechanism that drove these null responses (Perkowski *et al.*, 2021).

Second, we proposed an alternative hypothesis that negative effects of *Alliaria* presence would be strongest later in the growing season, as nutrient availability was expected to be reduced as the growing season progressed. Indeed, soil nitrogen availability and soil phosphorus availability were reduced in the second measurement period when compared to the first measurement period, which were associated with negative effects of *Alliaria* presence on photosynthetic capacity in *Trillium* spp. and stomatal conductance in *M. racemosum*. These patterns were associated with increased soil nitrogen: phosphorus ratios due to *Alliaria* presence early in the season that may have promoted greater reliance on disrupted AM fungal partners for soil resources. If true, increased reliance on AM fungal partners for phosphorus uptake may have been the mechanism that reduced nutrient provisioning to photosynthetic enzymes in *Trillium* spp. and reduced water uptake in *M. racemosum* later in the growth season.

*Photosynthetic responses to Alliaria presence are linked to altered nutrient and water economics*

*Alliaria* presence negatively impacted components of leaf-level photosynthesis in both species, consistent with our hypothesis. However, the mechanisms driving these responses were species-specific. Reduced apparent photosynthetic capacity in *Trillium* spp. suggests that *Alliaria* presence may have reduced nutrient provisioning toward the construction and maintenance of photosynthetic enzymes. Despite this, the null effect of *Alliaria* presence on relative chlorophyll content implies that reduced provisioning to photosynthetic enzymes was due to either a reduction in the fraction of leaf nutrients allocated to Rubisco or bioenergetics and was likely not a function of a change in the fraction of leaf nutrients allocated to light harvesting proteins (Niinemets & Tenhunen, 1997; Niinemets *et al.*, 1998; Waring *et al.*, 2023). While pools of leaf nitrogen content were not directly quantified here, stronger negative effects of *Alliaria* on the maximum rate of electron transport for RuBP regeneration suggests that *Alliaria* presence may have more strongly decreased the fraction of leaf nutrients allocated to light harvesting proteins than to Rubisco. Previous work indicates that glucosinolates can modify electron chain reactions (Rodgers *et al.*, 2008), but it remains unclear whether these patterns directly scale to modify provisioning of resources to photosynthetic tissues. Future work should aim to quantify these pools of leaf nutrients to better understand the mechanisms driving these responses.

*Maianthemum racemosum* responses to *Alliaria* presence suggests that the presence of this allelopathic invader induced a form of water stress, causing individuals to decrease stomatal conductance, reduce water use, and increase the extent by which stomatal conductance limited net photosynthesis. Reductions in water use did not correspond with reduced photosynthetic capacity, suggesting that *Alliaria* did not modify the nutrient economics of *M. racemosum*. These patterns support previous work showing that *M. racemosum* decreased net photosynthesis rates through decreased stomatal conductance and that these responses are not linked to nutrient limitation (Hale *et al.*, 2011, 2016). Instead, previous work has linked these responses to increased carbon limitation that increased *M. racemosum* leaf lifespan and reduced carbon storage (Hale *et al.*, 2016). These traits were not measured here, but could be a useful priority for future work to connect these responses with previous observations.

The two native species used in this study were expected to occupy similar niches in the plant community, yet had vastly different physiological responses to *Alliaria* invasion. Both species grew under the same aboveground and belowground environments, experienced the same *Alliaria* manipulations, form rhizomes and reproduce clonally, acquire nutrients and water through direct uptake pathways or symbioses with AM fungi, and have similar emergence times. It is possible that the different effects of *Alliaria* presence on leaf-level photosynthesis of *Trillium* spp. and *M. racemosum* may be linked to patterns expected through the leaf economics spectrum (Wright *et al.*, 2004; Reich, 2014). Shorter leaf lifespans in *Trillium* spp. individuals may cause individuals to be more closely aligned to resource acquisition strategies that foster rapid nutrient and water uptake to allow for more rapid growth and reproduction, while longer leaf lifespans in *M. racemosum* may cause individuals to fall closer to resource conservation strategies that foster longer-term investments toward growth and reproduction (Wright *et al.*, 2004). This pattern may explain why negative effects of *Alliaria* on *Trillium* spp. were driven by stronger changes in apparent photosynthetic capacity than changes in water use, as leaf nutrient demand to build and maintain photosynthetic enzymes may have been greater in *Trillium* spp. than *M. racemosum*. Previous work notes that negative effects of *Alliaria* presence on *M. racemosum* are often driven by changes in water use, which has been linked to increased leaf lifespans that are thought to be a compensatory mechanism to maximize carbohydrate storage (Hale *et al.*, 2016).

Effects of allelopathic invasion may differ among species with different leaf economic strategies. Specifically, species with extended growing seasons may experience stronger long-term negative outcomes of *Alliaria* invasion, as…

However, *Trillium* spp. have shorter leaf lifespans than *M. racemosum*. *Trillium* spp. leaves begin to senesce in June and *M. racemosum* leaves senesce as late as September (Heberling et al., 2019). Previous work noted that the carbon budgets of these two species are largely similar during the growing season, and any major differences in the seasonal carbon budgets may be attributed to differences in the timing of leaf phenology (Heberling et al., 2019).

*Using leaf physiology to assess linkages between aboveground and belowground responses to allelopathic plant invasion*

Native species’ physiological responses to *Alliaria* presence have direct implications for understanding the integrated negative effects of *Alliaria* invasion on belowground soil microbial community and aboveground plant community form and function. Previous work has demonstrated that *Alliaria* presence disrupts belowground AM fungal community composition (Callaway *et al.*, 2008; Anthony *et al.*, 2019). Similarly, *Alliaria* presence negatively affects the abundance and survivorship of AM native plants that coexist with *Alliaria* presence in its non-native range (Bialic-Murphy *et al.*, 2020; Roche *et al.*, 2021, 2023). Recent work has also highlighted that *Alliaria* presence may promote shifts in nutrient and water economics at the leaf-level (Bialic‐Murphy *et al.*, 2021) that may be a strategy that allows individuals to maximize carbon uptake at the lowest summed cost of using soil resources as expected from photosynthetic least-cost theory (Wright *et al.*, 2003).

Until now, limited work has focused on understanding the physiological mechanisms that drive the effects of *Alliaria* presence on native plant communities, and measurement periods have focused on timepoints where the tree canopy had already closed. The work presented here shows that *Alliaria* presence decreased net photosynthesis either by reducing apparent photosynthetic capacity or stomatal conductance, suggesting that *Alliaria* presence altered nutrient economies in *Trillium* spp. and water economies in *M. racemosum*. These patterns are directionally consistent with previously observed effects of *Alliaria* presence on belowground microbial community and aboveground plant community form and function, indicating that the impacts of *Alliaria* on below ground soil microbial communities are likely scalable to plant community dynamics.

*Conclusions*

Here, we show that *Alliaria* presence negatively affected leaf-level photosynthetic processes of two native AM understory perennial species. Negative effects of *Alliaria* presence on leaf-level photosynthesis in both species were generally stronger during the second measurement period after tree canopy closure and nutrient availability decreased. The negative effects of *Alliaria* presence on leaf-level photosynthesis in *Trillium* spp. were driven by reductions in apparent photosynthetic capacity that were counterbalanced by a nonsignificant increase in stomatal conductance. In contrast, the negative effects of *Alliaria* presence on leaf-level photosynthesis in *M. racemosum* were driven by reductions in stomatal conductance that increased stomatal limitation of net photosynthesis, but these responses were not associated with changes in apparent photosynthetic capacity. Photosynthetic responses to *Alliaria* presence in *Trillium* spp. may have been due to reduced nitrogen uptake and nitrogen allocation to photosynthetic leaf tissue, while net photosynthesis and stomatal conductance responses to *Alliaria* presence in *M. racemosum* may have been due to reduced water use that increased stomatal limitation. These results provide important insight into understanding the mechanisms that drive photosynthetic responses to allelopathic plant invasion and are a critical component needed to link effects of allelopathic plant invasion on belowground soil microbial communities to plant population and community dynamics.

**Author Contributions**

The sampling approach was the product of discussions between EAP, JM, LB-M, SNK, SK, and NGS. EAP conducted field work in collaboration with KC, JM, SC, and NGS. EAP conducted the analyses and wrote the first draft of the manuscript. Manuscript feedback was provided by all co-authors. The experiment has been maintained by SK since its inception. All authors support submission of this manuscript to XX.

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**Conflicts of Interest**

None.

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