

Regional Variation in *Phragmites australis* Reproductive Traits and Seedling Performance in North America

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Abstract

Seedling recruitment is an important mode of spread utilized by many invasive plants. In widespread invasive plants, regional variation in the rates of seedling recruitment can contribute to differences in invasion intensity across regions. In this study we examined regional variation in reproductive traits and seedling performance in a cosmopolitan invasive wetland grass, *Phragmites australis*. We tested whether nitrogen levels and regions with different histories and intensities of invasion would affect reproductive traits and seedling performance. We sampled invasive *Phragmites* inflorescences from 34 populations across three regions in North America: The Northeast (oldest most intense invasion), the Midwest (recent intense invasion), and Southeast (recent sparse invasion). We hypothesized that Northeast *Phragmites* populations would have the highest reproductive output and seedling performance, and that populations experiencing high nitrogen pollution would have higher reproductive output and seedling performance under high nitrogen conditions. We found that populations in the Northeast had the highest inflorescence mass, as expected. We also found that despite sparse distribution of *Phragmites* in the Southeast, populations from the Southeast displayed a high potential for sexual reproduction. However, increasing watershed-level nitrogen decreased percent seed germination in Southeastern populations, suggesting that Southeastern populations are sensitive to rising nitrogen levels. While elevated nitrogen increased belowground growth in Southeastern *Phragmites* seedlings, elevated nitrogen decreased belowground growth in Midwestern seedlings. These results suggest that the southeastern region of North America may become the next invasion front of *Phragmites*, warranting more research into the possible management of *Phragmites* spread in the region.

Introduction

Plants with cosmopolitan distributions often exhibit regional phenotypic variation across a variety of growth and reproductive traits (Weber & Schmid 1998; García-Nogales et al. 2016; McAssey et al. 2016; Xiao et al. 2019; Leal-Saénz et al, 2020). Due to founder effects and inbreeding, invasive plants can often have lower genetic diversity than native range populations (reviewed in Hernández-Espinosa et al. 2022). However, as the range of invasive plants expands, rapid evolution of growth and defense related traits also increases (reviewed in Felker-Quinn et al. 2013). Regional variation in traits aiding in expansion, invasiveness, and competitive ability can lead to invasion hotspots. Invasive plants such as *Spartina alterniflora* and *Phytolacca americana* display latitudinal variation in growth traits such as plant height and the culm/stem density which may contribute to their invasiveness in Eurasia (Liu et al. 2016; Xiao et al. 2019). Understanding regional variation in the traits of invasive species can allow us to better understand variation in invasive success and ultimately inform region-specific management plans.

Plant fecundity, germination, and early seedling performance are key traits that can determine invasiveness and that can exhibit regional variation. Fecundity traits can affect propagule pressure, which is one of the primary drivers in biological invasions (Colautti 2004; Simberloff 2010). For example, *S. alterniflora* (Liu et al. 2020), *Ambrosia artemesiifolia* (Zhou et al. 2021), *Ambrosia trifida* (Hovick et al. 2018), and *P. americana* (Xiao et al. 2018) exhibit regional variation in reproductive traits, which can lead to regional variation in seedling recruitment and differential rates of invasive expansion within the introduced range. Germination ability and seedling establishment and survival have also been found to be important characteristics of invasive plants (Pyšek and Richardson 2007). The seedling stage represents a

particularly vulnerable stage in a plant's life history, and a quick transition from relying on seed provisions to environmental sources of nutrients increases the chances of seedling survival (Leck et al. 2008; Silvertown 2008; Kettenring & Whigham 2018). Regional variation in germination and seedling performance has been observed across a variety of trees, forbs, and grasses and in both invasive and native plants (Jayasankar et al. 2003; Lamarque et al. 2015; Magni et al. 2019; Samis et al. 2019; Liu et al. 2020 Roman et al. 2022). One study also demonstrated that a species had higher seed provisioning in its invasive vs. native range, that translated into higher seedling performance in the invasive range (Hierro et al. 2013). Overall, understanding fecundity, germination, and early seedling performance across invasive ranges can help identify invasion hotspots and areas of potentially rapid expansion.

Global industrialization over the last 200 years has increased the rate of anthropogenic pollution resulting in excess deposition of nutrients into the environment (reviewed in Burkholder & Gilbert 2013). Anthropogenic nitrogen deposition is altering nitrogen dynamics in habitats worldwide, adding an additional layer of complexity to biological invasions (Canfield et al. 2010; Stevens et al. 2015). Increases in nitrogen can release plants from nutrient limitation and favor faster growing invasive plants that outcompete natives as seen with *Phalaris arundinacea* (Martina & Von Ende 2013) and *Molinia caerulea* (Tomassen et al. 2004). Wetlands often accumulate high concentrations of nitrogen from industrial and agricultural runoff and as a result can become areas of nitrogen saturation (Galloway et al. 2004; Withers et al. 2014; Fowler et al. 2015). This may explain why wetlands are disproportionately affected by biological invasions as they make up 6% of Earth's landmass but are home to 24% of the world's most invasive plants (Zedler & Kercher 2004). Because anthropogenic pollution is often linked

to biological invasions, understanding the nature of their relationship can inform current invasion dynamics and help us predict how they may change in the future.

Common reed, *Phragmites australis* (Cav.) Trin. ex Steud. is one of the most widespread plants on Earth and is found on every continent except for Antarctica (Packer et al 2017). In North America, the Eurasian lineage of *P. australis* (hereafter referred to as *Phragmites*) is rapidly becoming the most impactful invasive wetland plant (Chambers et al. 1999; Saltonstall 2002; Burdick & Konisky 2003; Buchsbaum et al. 2006; Lambertini et al. 2012). Although some studies suggest evidence of multiple introductions of *Phragmites* into North America (Saltonstall 2010; Meyerson & Cronin 2013), the earliest herbarium samples of *Phragmites* suggest that it was first introduced to the Mid-Atlantic region in the 19th century, and was first described in 1876, being found near ballast ground in Camden, New Jersey (Saltonstall 2002). *Phragmites* is now present across much of North America, but regional variation in distribution and invasion intensity exists (Saltonstall 2002; Kettenring et al. 2012). In the Northeast/Mid-Atlantic where the residence time of *Phragmites* is the longest, the invasion is also very intense, and evidence suggests most new populations are established through seedling recruitment (Saltonstall 2002; Kirk et al. 2011; Albert et al. 2015). In the Midwest, the invasion is relatively recent compared to the Northeast, but the intensity and impact of *Phragmites* on native wetlands has been mounting in recent years (Lynch & Saltonstall 2002; Wilcox et al. 2003; Price et al. 2014). While *Phragmites* has colonized as far as the west coast of North America and is becoming a problem in places like the Great Salt Lake in Utah (Kulmatiski et al. 2011; Long et al. 2017; EDDMapS 2024), the distribution of *Phragmites* in the Southeast and South-Central regions of the United States is rather sparse and patchy, and the invasion is comparatively benign (White et al. 2004; Meyerson et al. 2010; EDDMapS 2024). One common garden study found that

Phragmites growth tended to decrease with latitude, but flowering frequency tended to increase, suggesting that *Phragmites* fecundity may be even higher in southern populations (Mozdzer et al. 2016). Furthermore, environmental suitability modelling suggests that *Phragmites* is highly climatically suited to living in the Southeast and South-Central regions of the United States (Guo et al. 2013).

There is growing evidence that seedling recruitment is the primary mode of *Phragmites* expansion throughout much of North America. While *Phragmites* can disperse and establish new populations via vegetative propagules (rhizomes & culm fragments), these modes of dispersal are more stochastic and dependent on local hydrological conditions (Hudon et al. 2005; Meyerson et al. 2014). Studies suggest that the establishment of most new populations in the Northeast, Mid-Atlantic, and Midwest is through seedling recruitment (Brisson et al. 2010; Kirk et al. 2011; Albert et al. 2015; Kettenring et al. 2015; Fant et al. 2016). Once local genetic diversity is sufficient to overcome pollen limitation, *Phragmites* has a propensity for high sexual reproductive output (McCormick et al. 2010; Kettenring et al. 2011). *Phragmites* displays regional phenotypic variation in traits in both its invasive range in North American and its native range in Eurasia as common garden studies demonstrate variation in growth traits across regional scales (Clevering et al. 2001; Achenbach et al. 2012; Eller & Brix 2012; Ren et al. 2020).

Despite the importance of seedling recruitment in the establishment of new *Phragmites* populations in the Midwest, Northeast, and Mid-Atlantic, comparisons of *Phragmites* fecundity and seedling performance across regions have not been explored (Brisson et al. 2010; Kirk et al. 2011; Albert et al. 2015; Fant et al. 2016; Kettenring & Whigham 2018).

Phragmites thrives in high nitrogen environments, which often shifts competitive interactions in its favor when competing against native wetland plants (Minchinton & Bertness

2003; Rickey & Anderson 2004; Mozdzer & Zieman 2010; Mozdzer & Megonigal 2012). Elevated nitrogen can boost *Phragmites* fecundity by increasing the mass of individual inflorescences (Rickey & Anderson 2004), the number of inflorescences per plant, and the number of florets per inflorescence (Kettenring & Whigham 2009; Kettenring et al. 2011). Chronic nitrogen pollution may increase maternal seed provisioning and increase seed quality, as seen across commercial and wild grass species (Torres et al. 2009; Ronnenberg et al. 2011; Wang et al. 2022; but see Kettenring & Whigham 2009; Kettenring et al. 2011). Increased nitrogen availability promotes *Phragmites* seedling growth across a wide geographic sampling in Europe (Clevering 1999). Unlike European studies, only localized studies on the effects of nitrogen on *Phragmites* fecundity and seedling performance exist in North America.

Several studies have suggested that regional variation in genetic diversity exists in *Phragmites*. In the Northeast and Mid-Atlantic (Chesapeake Bay), high levels of genetic diversity have been described in local *Phragmites* populations, most likely due to multiple introductions of *Phragmites* into the area (Belzile et al. 2010; Kettenring et al. 2012). Studies in the Midwest find higher genetic diversity in the introduced lineage compared to the native lineage (Tipperry et al. 2020). This increase in genetic diversity is also correlated with the intensity of *Phragmites* invasion in the Midwest and the Atlantic, as *Phragmites* expansion in the Mid-Atlantic has increased by 25x over the last 40 years, and studies in the Saint Lawrence River and Rhode Island show that the introduced lineage has been expanding and dominating in native wetlands and estuaries (Lambert & Casagrande 2006; Belzile 2010; Kettenring et al. 2012). While there are multiple lineages of Eurasian *Phragmites* in the Mississippi river delta (White et al. 2004; Hauber et al. 2011), the distribution in the rest of the Southeast and South-Central region is rather sparse and the genetic diversity within the larger region is unknown. This

suggests that *Phragmites* in North America is likely not monomorphic and regional phenotypic variation may exist.

Here we determine the effects of *Phragmites* region of origin (Northeast, Midwest, and Southeast) and local watershed nitrogen load on *Phragmites* fecundity and germination by conducting a survey of reproductive traits across 34 populations in its invaded range in North America. Because of *Phragmites*' tendency to establish and flourish in nutrient enriched habitats, we hypothesize that H1.1) *Phragmites* populations experiencing **higher** nitrogen loads will have **greater** inflorescence length, inflorescence mass, spikelet number, average seed mass, percent germination, and reproductive potential (defined as spikelet number * percent germination) than populations experiencing lower nitrogen loads. The high intensity of *Phragmites* invasion in the Northeast is in some part due to the high rates of seedling recruitment; thus, we also hypothesize that H1.2) populations from the Northeast will have **greater** inflorescence length, inflorescence mass, spikelet number, average seed mass, percent germination, and reproductive potential than populations from the Southeast and Midwest.

Since seedling recruitment is determined by both fecundity and seedling establishment, we also examined variation in seedling growth by region and in response to elevated nitrogen by performing a seedling growth experiment using seeds collected from the Northeast, Midwest, and Southeast and grown under ambient and elevated nitrogen. We hypothesize that based on regional patterns of *Phragmites* invasion modes and seedling response to nitrogen H2.1) seedlings from the Northeast will have higher biomass, shoot height, and shoot number than seedlings from the Midwest and Southeast irrespective of nitrogen treatment, and H2.2) that elevated nitrogen will increase seedling growth, and this response will be more pronounced in populations with high local watershed nitrogen. In total, this research represents the first steps for

investigating the regional variation in seedling recruitment in *Phragmites* and will help predict the capacity for invasive spread of *Phragmites* in the future.

Methods

Sample collection

Inflorescences from 34 populations of *Phragmites* along a latitudinal gradient were collected between October - November 2021 (Figure 1; Table 1). Populations sampled spanned 44° N (Quebec, Canada) to 31° N (Brunswick, Georgia, USA), and from 69° W (Cape Cod, Massachusetts, USA) to 87° W (Johnsonville, Tennessee, USA) (Figure 1; Table 1). Sampling was conducted as follows: each collector would walk a 10m transect from the edge of the *Phragmites* stand and collect a single randomly selected inflorescence every meter for a total of 10 inflorescences per site (total N = 340). Each inflorescence was subsequently laid out in-lab at room temperature for 48 hours before being placed in an individual paper bag and then shipped and stored at room temperature at Tulane University before the start of processing in spring of 2022.

Seed & inflorescence traits

The length of individual inflorescences from each population were measured from the base of the inflorescence where the first branches emerge (junction between first pedicel and rachis) to the tip of the last spikelet. The mass of the inflorescence was obtained by measuring the full inflorescence and subtracting the mass of the seeds extracted from the inflorescence. An estimate of spikelet number per inflorescence for each sampled location was calculated by counting total spikelet number on two random inflorescences per population and using the

relationship between average spikelet number and mass to estimate spikelet number for the other eight inflorescences from that same population.

Seeds were extracted from each inflorescence by rubbing inflorescences against a 1mm sieve followed by a 0.5mm sieve. The chaff was blown off and only mature naked seeds were retained. An aliquot of 50 seeds was taken from each inflorescence and weighed on a microbalance (Mettler-Toledo; Columbus, OH, USA) to calculate mean seed mass.

Each 50-seed aliquot was plated on 1% agarose (50 seeds/ 90mm*15mm petri plate). All seeds were first sterilized using a modified version of the surface sterilization protocol from Shearin et al. (2018), consisting of a 3-minute 70% ethanol followed by a 3-minute 3% sodium hypochlorite wash on day 1, an overnight incubation at room temperature, and another round of surface sterilization on day 2 using a 5-minute ethanol wash followed by a 20-minute sodium hypochlorite wash. After the second surface sterilization wash, the seeds were placed in a growth chamber with a 18/6 hour light cycle and 30/15°C day:night temperature cycle. Petri dishes were checked daily for mold and all germinated seeds were counted after two weeks. Percent germination was calculated by counting germinated seeds per plate divided by 50. To estimate the possible reproductive output of *Phragmites* we created the metric, reproductive potential, which is the % germination multiplied by the number of spikelets. Number of seeds was not used as a fecundity metric, as phenological differences between populations prevented us from collecting all the inflorescences before seed drop occurred.

Seed Germination and Seedling Growth experiment

Of the 34 populations sampled, we chose a subset of 24 populations (indicated in Table 1) to use in the seedling growth experiment, due to seed stock limitations. These 24 populations

spanned at latitudinal range from Québec, Canada (N 46.832574°) to Brunswick, Georgia, USA (N 31.130608°) (Table 1). One seedling was grown from each of four randomly selected inflorescences collected from each of the 24 populations and was grown in two different nitrogen levels (elevated N; 25g.m².yr⁻¹, ambient N; 2g.m².yr⁻¹), totaling 192 pots (1 seedling * 4 inflorescences * 24 populations * 2 N treatments = 192 pots).

Seedlings for this experiment were germinated from 50-seed aliquots from four randomly selected inflorescences per population and germinated following the protocol described above. After one week, germinated seedlings were planted into square form pots (13.34cm L x 13.34cm W x 15.24cm H) filled with Leafgro high humus soil conditioner (Leafgro, Maryland Environmental Service, Millersville, Maryland, USA). Pots were placed in flat trays (54.43 cm L x 27.79 cm W x 20 cm H), each holding up to 8 pots, at which time the trays were fertilized with either the elevated N treatment or the ambient N treatment. The programming for the growth chamber light and temperature regime were taken as the average day length in May (14/10 hour light cycle) and a 25/15°C day:night temperature cycle, representative of the mid-latitude (N 38°) climate. The amount of photosynthetically active radiation (PAR) at soil level was 250 umol/m²/s (for reference full sunlight ~2000 umol/m²/s). Trays were bottom watered to the lip of the tray twice per week and the corresponding N treatments were added to the trays every 2 weeks, at which time pots were rotated between trays receiving the same nitrogen treatment.

Seedling height and shoot number were collected throughout the growth experiment on days 7, 14, 28, and 42. Shoot number and shoot height within each pot was collected by counting the number of fully emergent shoots over 5mm in height. Seedling harvest commenced on day 56. Seedlings were clipped at the soil line and the third fully formed leaf of the tallest

shoot within each pot was collected for specific leaf area (SLA) measurements. Freshly collected leaves were then photographed next to a ruler for scale, converted to 8-bit black and white images and leaf area was measured using ImageJ (Schneider et al. 2012) area calculations based on scaled photographs of the SLA leaf sample. Both the SLA leaf sample and the rest of the aboveground biomass were dried separately to constant mass at 50°C. SLA was then calculated by dividing dry mass of the SLA leaf sample by the ImageJ measured area of the corresponding leaf. Belowground biomass was washed clean of soil using a 1mm sieve to retain detached fine roots, before belowground biomass was dried to constant mass at 50°C. A subset of the roots was stored for up to 7 days at 4°C while root imaging was performed prior to drying. Root images were processed using the Rhizovision software (Seethepalli & York 2020) to derive root length by root class (fine roots (<1mm), secondary roots (1-3mm), and rhizome (>3mm)), specific root length, rooting depth, and root volume by root class.

Nitrogen and climate data

Total nitrogen loads (kg/km²) from the watershed corresponding to each site were obtained using datasets from the USGS SPARROW mapper (Ator 2020) which shows the annual nitrogen load in 2012. Mean growing season (March-October) temperature and precipitation were extracted from the NOAA National Centers for Environmental Information climate monitoring data which displays temperature and precipitation on the county level. Climatic metrics were collected to estimate the relative influence of climate on the observed fecundity traits.

Statistical analysis

To test the effects of region (Northeast, Midwest, and Southeast) and watershed level nitrogen on each of our response variables in the field survey (inflorescence length, inflorescence mass, spikelet number, average seed mass, and germination rate) we compared multiple linear mixed effects models and generalized least square models using the “lme” and “gls” functions in the R package “nlme” version 3.1-149 (Pinheiro et al. 2023) in R studio version 1.3.1093 (R Core Team 2023). We first created a base model for backwards selection by including all fixed effects (region*watershed-level nitrogen + temperature + precipitation) in a model explaining variance in a particular response variable. Population was used as a random effect in all models. Backwards selection was performed, and best-fit models were selected using AIC. Because populations displayed a wide range of variance, we tested for heterogeneous variances across different populations using AIC. We then ran a type III ANOVA on the best model to determine the significance of each of the explanatory variables. We performed model validation by plotting fitted values against residuals, each of the fixed effects against residuals, and a histogram of the residuals alone. Because germination rates are proportions, we used binomial regression models using the “glm” function in R package “stat” version 4.0.3 (R Core Team 2023). Binomial regression models did not include the random effect and heterogeneous variances of population due to constraints of the “glm” function.

For the seedling growth experiment, the effect of watershed level nitrogen* region* nitrogen treatment were used as fixed effects in models of seedling growth response variables (total biomass, aboveground biomass, belowground biomass, aboveground:belowground biomass, SLA, total shoot height, shoot number, total root volume, total root length, specific root length, root depth, and the volume and length of each root class (fine roots (<1mm Ø), secondary roots (1-3mm Ø), and rhizomes (>3mm Ø)) using site as a random effect in multiple linear

mixed effects models described above. Tukey's HSD test was performed on significant fixed effects to elucidate significant pairwise differences among factor-levels within each categorical factor using the "lsmeans" function in the R package "lsmeans" (Lenth 2018) to extract least-square means and then using the extracted means to perform a Tukey's HSD test in compact letter design using the "cld" function in the R package "multcomp" (Hothorn et al. 2023). In the case of significant interactions between fixed effects, a new model was run combining the two fixed effects into a single factor so that the Tukey's test could be performed.

Results

Inflorescence and Seed traits

Inflorescence mass was significantly influenced by region (Table 2). Inflorescences from plants from the Northeast ($3.68 \text{ mg} \pm 0.24$) had 22% and 7.6% more mass than inflorescences from plants from the Midwest ($3.15 \text{ mg} \pm 0.20$) and Southeast ($3.58 \text{ mg} \pm 0.27$) respectively, although the Tukey's HSD post-hoc test was unable to tease apart significant pairwise differences between regions (Figure 2A). Inflorescence length and the number of spikelets on each inflorescence were not significantly related to local watershed nitrogen levels or region (Table 2, Figure 2B-C).

None of our explanatory variables significantly explained mean seed mass (Table 2). However, the effect of local watershed nitrogen on percent germination depended on region (Table 2) (significant region x watershed nitrogen interaction), such that there was an inverse relationship between watershed nitrogen levels and percent germination only in populations sourced from the Southeast ($R^2 = 0.406$) (Figure 3). Regions differed significantly in

reproductive potential (Table 2), as plants from the Southeast (3623.99 ± 357.58) had 33% higher reproductive potential than plants from the Northeast (2721.94 ± 219.38) (Figure 2F).

Seedling growth traits

The effect of nitrogen on total shoot height and shoot number after 42 days of growth was dependent on region (significant N x region interaction) (Table 3). Tukey's HSD post-hoc test was unable to parse pairwise differences between treatment groups for total shoot height; however, Southeastern *Phragmites* responded positively to elevated nitrogen, with seedlings accumulating 22% more shoot height ($2516.06 \text{ mm} \pm 252.96$) than seedlings grown under ambient conditions ($2070.88 \text{ mm} \pm 198.01$) (Figure 4A). Conversely, seedlings from the Midwest had a negative response to elevated nitrogen, with seedlings grown under ambient nitrogen ($2177.71 \text{ mm} \pm 136.034$) accumulating 12% more shoot height than seedlings grown under elevated nitrogen ($1946.68 \text{ mm} \pm 97.58$) (Figure 4A). For shoot number, seedlings from the Southeast grown in elevated N (5.94 ± 0.504) had significantly more shoots (38%) than the same seedlings grown under ambient N (4.31 ± 0.35), whereas elevated nitrogen did not affect shoot number in Northeast and Midwest populations (Figure 4B).

The effect on nitrogen on total and belowground biomass also depended on region (significant N x region interaction) (Table 3). Although Tukey's HSD post-hoc test was unable to resolve significant pairwise differences, there were notable patterns in total biomass between the treatment groups. Namely, Southeastern *Phragmites* seedlings grown under elevated nitrogen ($2.49 \text{ g} \pm 0.33$) accumulated 31% more total biomass than seedlings grown under ambient conditions ($1.90 \text{ g} \pm 0.23$) (Figure 4C). The opposite pattern was observed in seedlings from the Midwest as seedlings grown under ambient nitrogen ($1.90 \text{ g} \pm 0.18$) accumulated 16% more total

biomass than seedlings grown under elevated nitrogen ($1.648 \text{ g} \pm 0.128$) (Figure 4C). The belowground biomass of seedlings from the Southeast grown under elevated nitrogen ($0.69 \text{ g} \pm 0.10$) was 63% higher than that of Midwest seedlings under the same conditions ($0.42 \text{ g} \pm 0.04$) (Figure 4C). There was a trend that the effect of nitrogen on aboveground biomass also depended on region (nearly significant N x region interaction) (Table 3); nitrogen only increased aboveground biomass of seedlings from the Southeast (Figure 4C). The effect of nitrogen on aboveground:belowground biomass ratio was also dependent on region (significant N x region interaction) (Table 3). Seedlings from the Southeast grown under ambient nitrogen (2.59 ± 0.24) had 20% lower aboveground:belowground biomass ratio than seedlings from the Midwest grown under elevated nitrogen (3.24 ± 0.19) (Figure 4D). Watershed-level nitrogen did not explain total ($F_{1,173} = 0.021$, n.s.), aboveground ($F_{1,173} = 0.077$, n.s.), or belowground ($F_{1,173} = 0.008$, n.s.) biomass under elevated and ambient nitrogen.

The only root traits significantly influenced by any of our explanatory variables were specific root length and secondary root volume, which varied significantly depending on region (Table 4). Seedlings from the Southeast ($10,936.18 \text{ mm}^3 \pm 1602.86$) had 68% higher secondary root volume than seedlings from the Midwest ($6,527.24 \text{ mm}^3 \pm 500.90$) (Figure 5A) and seedlings from the Northeast ($28.56 \text{ mm/mg} \pm 1.54$) had 17% higher specific root length than seedlings from the Midwest ($24.44 \text{ mm/mg} \pm 1.17$) (Figure 5B).

Discussion

The effects of local watershed nitrogen on seed germination

We hypothesized that *Phragmites* populations experiencing higher watershed N loads would have higher fecundity and germination. However, we found that in general watershed-level nitrogen did not affect any of the inflorescence traits, average seed mass, or reproductive potential. We did find that watershed N only affected germination rates in the Southeast, where higher N decreased germination.

Phragmites populations from the Southeast were more sensitive to local variation in nitrogen loads than in other regions, at least with respect to percent germination. While regional variation in germination success in other plant species has been observed (García-Nogales et al. 2016; Mohl et al. 2023), few studies have related variation in germination success to maternal nitrogen exposure. One study found that Powell amaranth (*Amaranthus powellii*), a noxious weed in croplands, experiences lower germination success in populations exposed to conventional (inorganic N) vs. organic (organic N) fertilization practices (Brainard et al. 2006). Another study found that in the yew species *Taxus baccata*, long-term maternal nitrogen fertilization reduced percent germination in offspring (Pers-Kamczyc & Suszka 2022). These studies are not in line with what we observe in *Phragmites*, where more nutrient polluted regions (Northeast & Midwest) are less sensitive to local nitrogen levels than the historically less-polluted Southeast. Several experimental studies show that nitrogen fertilization decreases seed viability in *Succisa pratensis* (Vergeer et al. 2003), *Juniperus communis* (Gruwez et al. 2014), and a variety of subarctic plants (Milbau et al. 2017). However, nitrate can increase seed viability (Ronnenberg et al. 2011; Baskin & Baskin 2014), but studies in other grass species generally find no effect on seed viability (Wagner et al. 2001; Torres et al. 2009 Tullos & Cadenasso 2016). Notably, our *Phragmites* populations from the Southeast experienced on average lower watershed-level nitrogen ($1.257\text{g/m}^2/\text{yr} \pm 0.073$) than the Northeast ($3.145\text{g/m}^2/\text{yr} \pm 0.335$) or the

Midwest ($1.761\text{g/m}^2/\text{yr} \pm 0.0908$). This suggests that either the Southeast experiences lower levels of nitrogen pollution or that perhaps populations there are not adapted to high nitrogen levels. Like what has been observed in other grass species, localized studies in the Chesapeake have found that elevated nitrogen had no effect on *Phragmites* seed viability (Kettenring et al. 2011). However, to our knowledge, studies on the effects of nitrogen pollution in other regions have not yet been explored. It is possible that low seed germination rates of Southeastern *Phragmites* populations established in nitrogen polluted areas may dampen its invasiveness in those areas, and this warrants more research into whether nitrogen pollution is influencing *Phragmites* invasion dynamics across different regions.

Regional variation in reproductive traits

In line with our predictions, inflorescences from the Northeast had higher mass than those from the Southeast and Midwest, even after accounting for weight of seeds. Increased inflorescence mass suggests that the plants from the Northeast are investing more resources into reproduction than other regions (Mullins and Marks, 1987). Considerable intraspecific variation in reproductive investment exists globally across various lineages of *Phragmites*, but the North American invasive *Phragmites* allocates the most to reproduction compared to populations from Europe and Asia (Pyšek et al. 2019). The increased inflorescence mass of Northeast populations mirrors the wide distribution and intensity of *Phragmites* invasion in the Northeast (Saltonstall 2002; Burdick & Konisky 2003; Kiviat 2010; EDDMaps 2024). This points to increased reproductive investment as a potential mechanism for its dominance in the Northeast, and warrants further research linking seedling recruitment and fecundity in the region,

While inflorescence mass varied regionally according to our hypothesis, seed traits (seed mass, germination, reproductive potential) did not demonstrate regional variation. The average seed mass from populations from the Northeast was the same as populations from the Midwest and Southeast. Furthermore, Northeast seeds had the lowest percent germination of any region, even considering the significant decrease in percent germination by Southeast populations with high levels of maternal N exposure. While regional variation in percent germination has not previously been studied in *Phragmites*, such variation has been observed in a variety of other invasive plant species. For example, invasive *Ambrosia artemisiifolia* in China exhibited lower germination in seeds from Northeastern latitude populations versus Southeast populations (Zhou et al. 2021). Invasive *Spartina alterniflora* populations in China displayed the opposite pattern, with populations in Northeastern China exhibiting a higher percent germination than populations from the Southeast (Cheng et al 2022). Thus, our results show that *Phragmites* populations from the Northeast invested more into inflorescences, but this investment did not translate into increased seed mass or germination. The reproductive potential of *Phragmites* from the Southeast was significantly higher than populations from the Northeast, which is opposite to our hypothesized patterns. These results suggest that seedling recruitment in the Southeast would be higher than in the Northeast. *Phragmites* habitat suitability modelling indicates that *Phragmites* can readily establish in the Southeast and South-Central regions of North America, yet the distribution of *Phragmites* in this region is sparse (Guo et al. 2013; EDDMapS 2024). Interestingly, a common garden study found that *Phragmites* grown in low-latitude climates tended to produce more inflorescence per plant, suggesting higher reproductive output in the South which is in-line with our findings (Mozdzer et al. 2016). Human activity (human population density, land use, and human access) is the strongest driver in the genetic structuring

of *Phragmites* populations in North America (Guo et al. 2018). Human activity can increase disturbance and nutrient pollution, both of which have been linked to increased invasion in *Phragmites* (Chambers et al. 1999; Bertness et al. 2002; Minchinton & Bertness 2003; Silliman & Bertness 2004). Therefore, the spread of *Phragmites* may potentially be due to the history of industrialization in North America, which has historically lagged in the South (Bateman & Weiss 1975; Guo et al. 2018). Several studies show that weak Allee effects can limit the local spread of *Phragmites*, as local genetic diversity must accumulate for outcrossing to occur and produce viable seed (McCormick et al. 2010; Kettenring et al. 2011). Although we cannot accurately determine the number of seeds produced on each inflorescence, it is notable that populations from the Southeast had a lower number of seeds than the other regions we sampled. Because our reproductive potential metric only includes spikelet number x germination rate, and spikelets can have variable numbers of seeds, our metric misses important information on seed set which is where Allee effects would manifest. More research is warranted into studying the dynamics of reproduction and identifying potential limits in seedling recruitment in the Southeast, as we found the potential for high seedling recruitment.

Regional variation in seedling response to nitrogen

We uncover inverse belowground seedling responses to elevated nitrogen between *Phragmites* populations in the Southeast and Midwest. Elevated nitrogen boosted belowground biomass in seedlings from the Southeast while elevated nitrogen decreased belowground biomass in seedlings from the Midwest. This pattern may be driven by differences observed in secondary root volume, as seedlings from the Midwest had significantly lower root volume than plants from the Southeast. As a result of the inhibitory effects of elevated nitrogen on belowground growth in

Midwestern seedlings, these seedlings had the highest aboveground to belowground biomass ratio observed in our study.

Ammonium (NH_4) and nitrate (NO_3) can act as signals for the proliferation or inhibition of lateral root growth, with both low N and high N thresholds for lateral root growth inhibition reported (Zhang et al. 1999; 2007). Other studies have also demonstrated a negative, neutral, and even positive relationships between sediment nitrogen levels and *Phragmites* belowground biomass, suggesting that there is considerable intraspecific variation in responses to nitrogen fertilization (Holdredge et al. 2010; Mozdzer & Megonigal 2012; Caplan et al. 2014; Chen et al. 2017; Gonzalez Mateu et al. 2021). These results suggest that the consequences of eutrophication on *Phragmites* belowground biomass may be very different from region to region. Elevated nitrogen decreased the belowground biomass of the native wetland grass *Spartina alterniflora* and is hypothesized to cause tidal marsh collapse through increased tidal erosion in *Spartina*-dominated marsh (Deegan et al. 2012; Hanley et al. 2021). This phenomenon may very well be reflected in *Phragmites*, and if so, increased eutrophication in Midwestern (but not Northeastern or Southeastern) *Phragmites* dominated wetlands may lead to marsh destabilization. Increases in belowground biomass may also increase the priming of deep carbon pools as *Phragmites* can increase soil aeration through its aerenchyma tissue leading to higher rates of decomposition and subsidence (Bernal et al. 2017, Bernal et al 2023).

Seedlings originating from the Southeast experienced a significant increase in shoot number when grown under elevated versus ambient nitrogen. Even though region*nitrogen treatment differences could not be resolved in the post-hoc test, total shoot height tended to increase with nitrogen fertilization in the Northeast and Southeast seedlings but decrease in the Midwest. Although the expansion of *Phragmites* stands into adjacent wetland can be achieved

through vegetative lateral expansion (Güsewell & Edwards 1999; Minchinton & Bertness 2003; Brisson et al. 2010), recent work has revealed that the advancing front of *Phragmites* stands can often be composed of multiple genets, highlighting the role of seedling recruitment in *Phragmites* patch expansion (Kettenring et al. 2016). Increased density in mature *Phragmites* stands correlates to lower native plant biomass and diversity (Holdredge & Bertness 2011; Elsey-Quirk & Leck 2021 but see Theuerkauf et al. 2017). Thus, increased shoot density of seedlings recruited near the edge of *Phragmites* stands may help in outcompeting native plants at the interface between native wetlands and invasive *Phragmites*. While seedling shoot density and biomass may not affect native plant communities as much as mature stands do, increased seedling vigor can help overcome seedling-stage mortality associated with flooding and competition and aid in successful establishment (Mauchamp et al. 2001; Saltonstall & Stevenson 2007).

Taken together, our results suggest that regional variation in seedling responses to nitrogen exists, as populations from the Southeast responded positively to increased nitrogen fertilization while Midwest populations showed the opposite pattern. Increased anthropogenic nutrient pollution may influence different regions in different ways and potentially promote the invasion of populations in the Southeast more than other regions. Regional variation in belowground patterns in response to nitrogen may also influence important wetland characteristics such as marsh integrity and rates of soil decomposition.

Conclusions

Here we demonstrated a high degree of intraspecific variation in fecundity and seedling performance in *Phragmites* across its invaded range in North America. There is a disconnect between the relatively low abundance of *Phragmites* in the Southeast and the high reproductive potential and high growth rates of Southeastern seedlings observed in this experiment. More research is needed to elucidate the mechanisms inhibiting Southeast *Phragmites* spread within the region. Anthropogenic nitrogen pollution has differential effects on belowground investment and shoot number of *Phragmites*. The differential belowground effects of elevated nitrogen in the Southeast versus the Midwest may have cascading effects in marsh stability and soil decomposition and may influence the ability of *Phragmites* dominated marshes in the Southeast to keep up with sea-level rise. Additionally, an increase in soil nitrogen promoted high seedling growth rate in Southeastern *Phragmites* which may increase the rate of seedling recruitment. Thus, southeastern *Phragmites* populations represent a potential invasion hotspot in the future, which warrants further exploration into the factors limiting invasion in this region. This current study along with future research will help educate management in ways of combatting further spread of *Phragmites* into the Southeast through understanding regional invasion dynamics in *Phragmites*.

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Authorship contribution: S.L., K.C., M.K.M, T.J.M., and E.C.F. in conjunction designed the experiment. S.L. and volunteers helped with sampling of *P. australis* material. All procedures and data collection were performed by S.L. Guidance and assistance in data analysis was offered by E.C.F. Data analyses were performed by S.L. First draft was written by S.L. and all authors edited the manuscript.

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Data availability: The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Tables

Table 1 Region, site name/ city/ state/province/ country of origin, coordinates, and watershed-level nitrogen of each *Phragmites* population used to source inflorescences and seed for inflorescence/seed trait analysis and seedling growth experiment (indicated by the *).

Region	Site (City, State/Province, Country)	Latitude	Longitude	Watershed-level nitrogen (kg/km ² /year)
Northeast	Québec City, QC, Canada *	46.832574	-	3.700
Northeast	Blainville 1, QC, Canada *	45.661025	-	8.176
Northeast	Blainville 2, QC, Canada *	45.661025	-	8.176
Northeast	Saco, ME, USA *	43.488616	-	0.366
Northeast	Biddeford, ME, USA	43.457593	-	0.366
Northeast	Rowley, MA, USA *	42.724972	-	3.421
Northeast	Boston, MA, USA *	42.343408	-	1.584
Northeast	Wellfleet, MA, USA *	41.902449	-	0.226
Northeast	Westport, CT, USA *	41.117198	-	1.550
Northeast	North Brunswick, NJ, USA	40.473528	-	2.123
Northeast	Hamilton, NJ, USA	40.230546	-	2.123
Northeast	Villanova, PA, USA	40.041639	-	1.979
Northeast	Philadelphia, PA, USA *	39.890278	-	9.932
Midwest	Novi, MI, USA *	42.487883	-	4.481
Midwest	La Porte, IN, USA *	41.691709	-	1.245
Midwest	Schererville, IN, USA	41.506281	-	4.531
Midwest	Lafayette, IN, USA *	40.446970	-	2.407
Midwest	Indianapolis, IN, USA *	39.791320	-	2.901
Midwest	Patricksburg, IN, USA	39.276672	-	1.843
Midwest	Lincoln City, IN, USA *	38.133506	-	2.073
Midwest	Henderson, KY, USA *	37.866148	-	1.644
Midwest	Central City, KY, USA *	37.289160	-	2.002
Midwest	Muhlenberg, KY, USA *	37.275330	-	2.002

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	Midwest	Graham, KY, USA	37.250730	87.246450	2.002
550				-	
551	Midwest	McNary 1, KY, USA *	37.236570	87.327350	2.002
552				-	
553	Midwest	McNary 2, KY, USA *	37.234950	87.350610	2.002
554				-	
555	Midwest	New Johnsonville, TN, USA *	36.035178	87.987878	0.598
556				-	
557	Southeast	Gloucester Point, VA, USA *	37.246361	76.504528	0.565
558				-	
559	Southeast	Kill Devil Hills, NC, USA	36.004500	75.673667	0.845
560				-	
561	Southeast	Wanchese, NC, USA *	35.870833	75.642694	1.190
562				-	
563	Southeast	Georgetown, SC, USA *	33.258353	79.255025	0.843
564				-	
565	Southeast	Savannah, GA, USA	32.071778	81.076806	2.910
566				-	
567	Southeast	Broadfield, GA, USA	31.326252	81.445617	1.345
568				-	
569	Southeast	Brunswick, GA, USA *	31.130608	81.479963	1.451

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Table 2 ANOVA degrees of freedom, F-value, and associated p-value from linear mixed effect models testing the effect of local watershed level nitrogen * region and environmental factors (precipitation and temperature) on inflorescence and seed traits collected from North American *Phragmites* in 2021.

	Inflorescence Mass			Inflorescence Length			Spikelet Number		
	numDF	F-value	p-value	numDF	F-value	P-value	numDF	F-value	p-value
Nitrogen	1	0.376	0.513	1	0.067	0.798	1	0.309	0.583
Region	2	3.704	0.038	2	0.288	0.752	2	2.215	0.130
Region:Nitrogen	2	2.211	0.129	2	0.265	0.769	2	0.419	0.662
	Mean Seed Mass			Percent germination			Reproductive potential		
	numDF	F-value	p-value	numDF	Chi-squared	p-value	numDF	F-value	p-value
Nitrogen	2	1.889	0.309	2	12.424	0.001	2	3.995	0.205
Region	1	1.080	0.172	1	10.211	0.002	1	1.700	0.032
Region:Nitrogen	2	0.407	0.670	2	11.767	0.003	2	1.526	0.239

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629 **Table 3** ANOVA numerator degrees of freedom, F-value, and associated p-value from linear mixed effect models testing the
630 effect of nitrogen * region on seedling growth traits and biomass collected from *Phragmites* seedling growth experiment. P-value
631 significance is reported, p <0.05(*), p <0.01(**), p<0.001(***)

	Total biomass			Aboveground biomass			Belowground biomass			Aboveground: Belowground Biomass		
	DF	F-val	p-val	DF	F-val	p-val	DF	F-val	p-val	DF	F-val	p-val
Region	2	1.053	0.367	2	0.900	0.422	2	1.247	0.308	2	2.700	0.090
Nitrogen	1	5.645	0.019 (*)	1	7.573	0.007 (**)	1	0.023	0.879	1	1.433	0.233
Region:nitrogen	2	3.456	0.034 (*)	2	2.500	0.086	2	3.625	0.029 (*)	2	4.072	0.019 (*)

	Specific Leaf Area			Total Shoot Height (day 42)			Shoot Number (day 42)		
	DF	F-val	p-val	DF	F-val	p-val	DF	F-val	p-val
Region	2	0.509	0.608	2	0.401	0.674	2	0.026	0.975
Nitrogen	1	1.092	0.298	1	2.619	0.108	1	5.878	0.017 (*)
Region:nitrogen	2	0.023	0.977	2	3.444	0.034 (*)	2	3.976	0.021 (*)

Table 4 ANOVA degrees of freedom, F-value, and associated p-value from linear mixed effect models testing the effect of nitrogen * region on root traits collected from *Phragmites* seedling growth experiment.

Total Root Length				Fine Root Length		
	numDF	F-value	p-value	numDF	F-value	p-value
Region	2	3.162	0.063	2	3.131	0.065
Nitrogen	1	0.090	0.765	1	0.573	0.452
Region:nitrogen	2	0.731	0.485	2	0.898	0.413
Secondary Root Length				Rhizome Length		
	numDF	F-value	p-value	numDF	F-value	p-value
Region	2	3.030	0.070	2	1.919	0.172
Nitrogen	1	0.634	0.429	1	0.001	0.970
Region:nitrogen	2	0.217	0.806	2	0.679	0.511
Total Root Volume				Fine Root Volume		
	numDF	F-value	p-value	numDF	F-value	p-value
Region	2	2.696	0.091	2	2.669	0.093
Nitrogen	1	0.004	0.951	1	0.030	0.864
Region:nitrogen	2	0.451	0.639	2	0.977	0.382
Secondary Root Volume				Rhizome Volume		
	numDF	F-value	p-value	numDF	F-value	p-value
Region	2	3.624	0.045	2	1.587	0.228
Nitrogen	1	0.297	0.588	1	0.392	0.533
Region:nitrogen	2	0.444	0.644	2	2.229	0.116
Specific Root Length				Root Depth		
	numDF	F-value	p-value	numDF	F-value	p-value
Region	2	4.823	0.019	2	0.899	0.422
Nitrogen	1	1.586	0.212	1	1.061	0.307
Region:nitrogen	2	3.089	0.052	2	0.862	0.428

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Figure 1 Location of *Phragmites* populations used for inflorescences collections in 2021. The points correspond to location of sites, the green points indicate populations designated as Northeast populations, blue points indicate populations designated as Southeast populations, and red points indicate populations designated as Midwest populations.

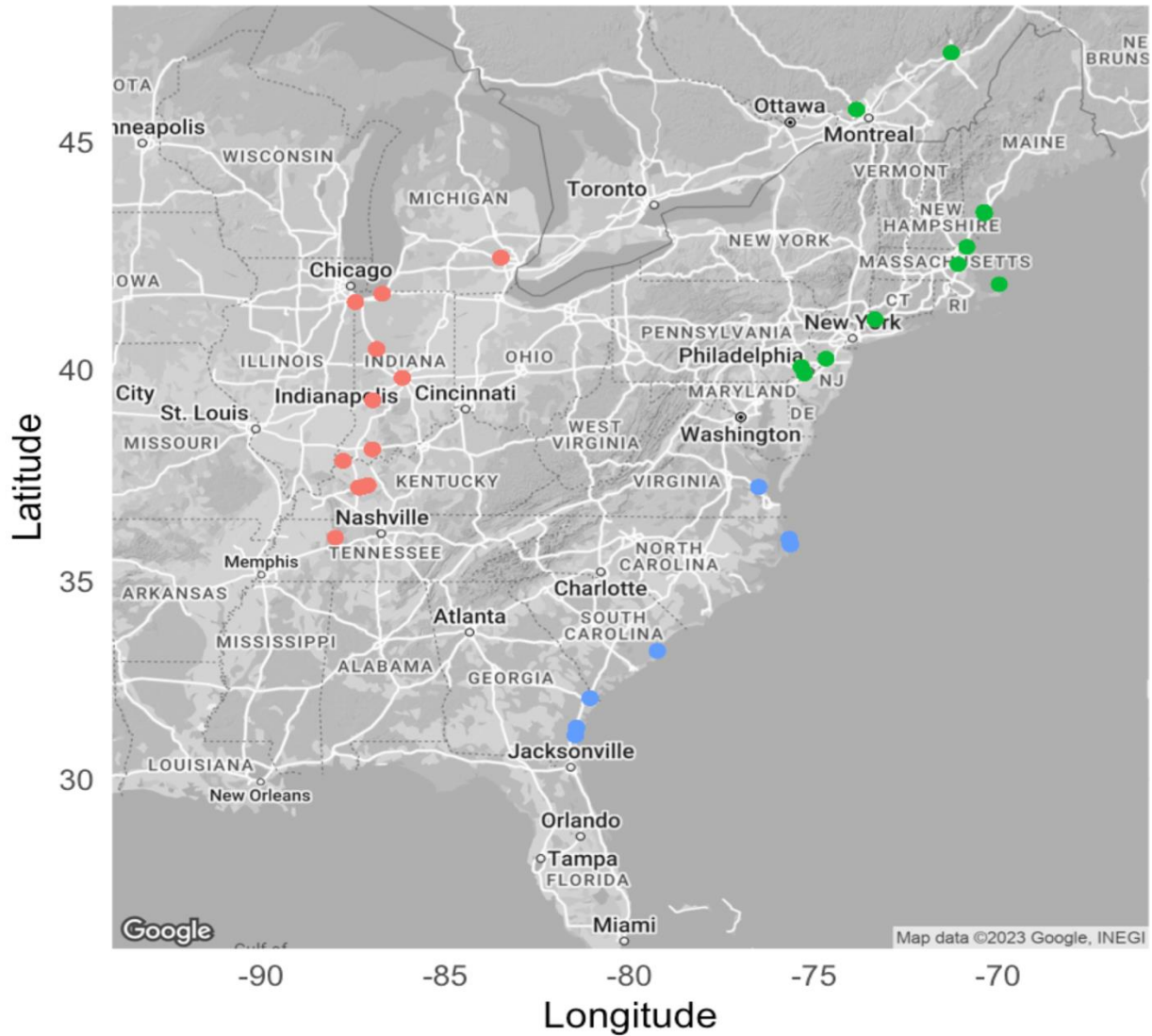


Figure 2 Mean of A) inflorescence mass, B) inflorescence mass, C) number of spikelets, D) average seed mass, E) percent germination, and F) reproductive potential by region of origin (Northeast, Midwest, and Southeast). Letters represent Tukey's HSD test displayed in compact letter design (CLD) showing significant pairwise comparisons between each region. The dark red diamond within each boxplot (A-D, F) represents the mean value, the upper and lower hinges of each boxplot represent the 25th and 75th quantile, the middle line represents the median value, the whiskers represent the 5th and 95th percentile, and outliers are represented by black points. The box and whiskers in the violin plot represent the 10th quantile (lower whisker), 25th quartile (lower edge of box), midline (median), 75th quartile (top edge of box), and 90th quantile (top whisker).

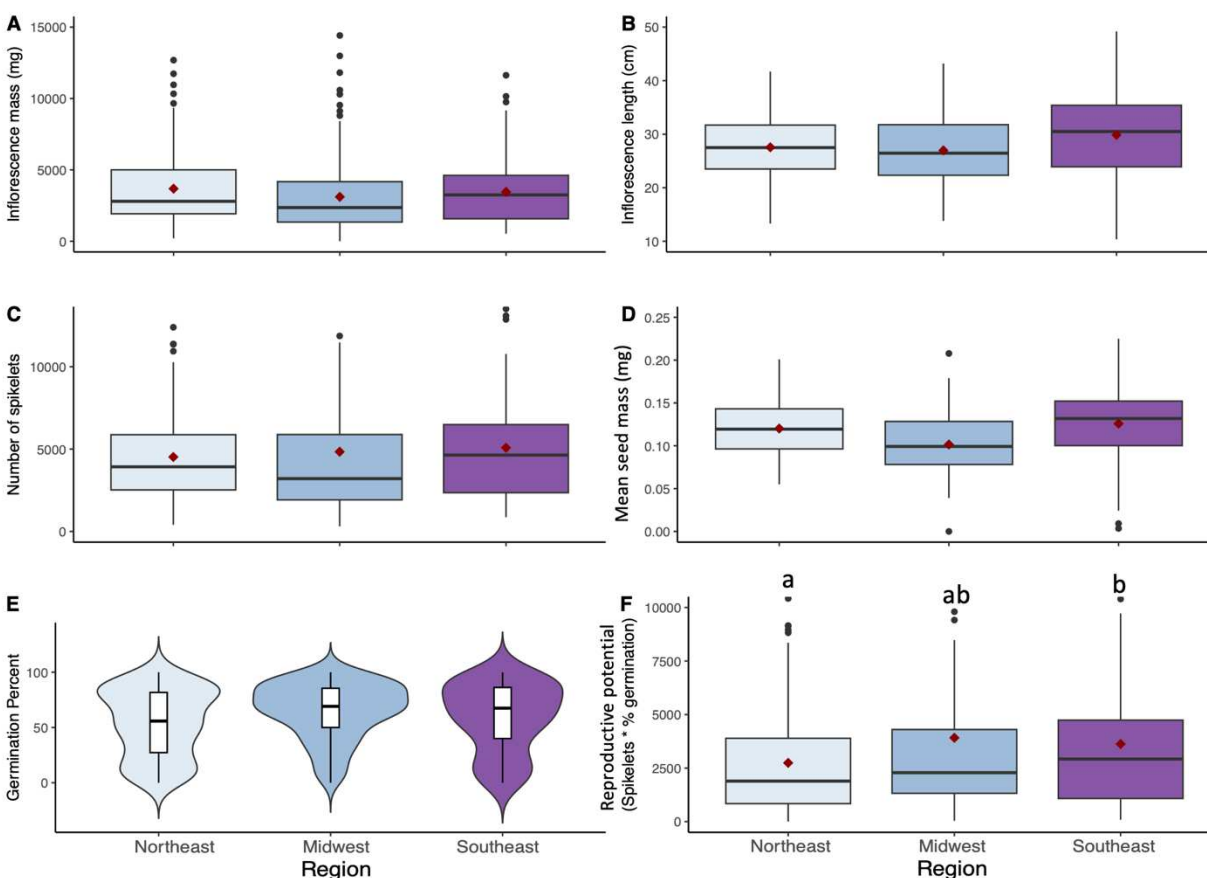


Figure 3 Effect of logarithm of watershed level nitrogen on percent germination across region of origin (Northeast, Midwest, and Southeast). Green points represent seeds from inflorescences sourced from the Northeast, blue points represent seeds from inflorescences sourced from the Southeast, and red points represent seeds from inflorescences sourced from the Midwest. Lines represent local regression and grey area represents the confidence interval.

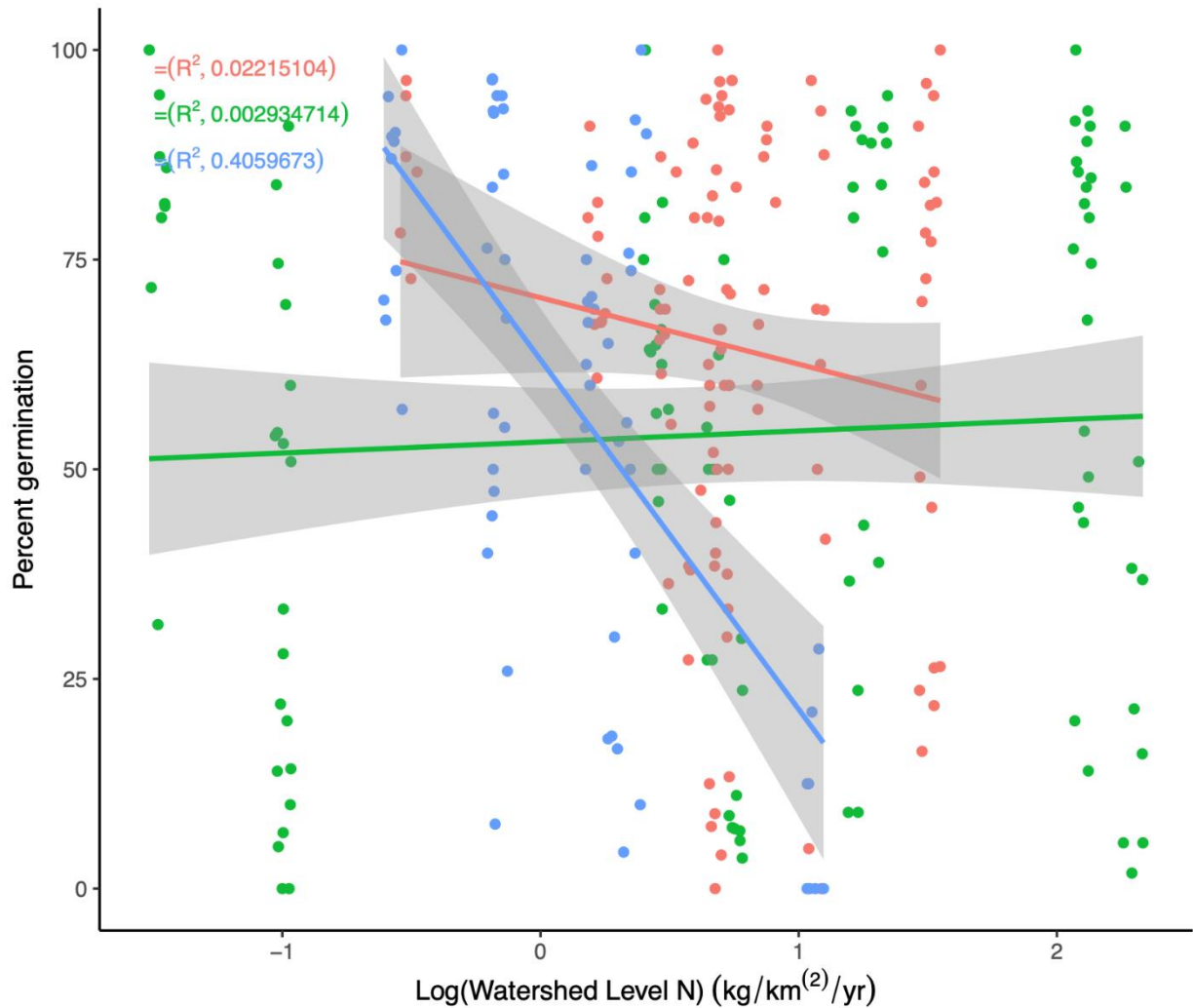


Figure 4 Mean of A) total shoot height at day 42, B) number of shoots at day 42, C) aboveground and belowground biomass, and D) aboveground:belowground biomass by region of origin (Northeast, Midwest, and Southeast) and nitrogen treatment (ambient and elevated nitrogen). Letters represent Tukey's HSD test displayed in compact letter design (CLD) showing significant pairwise comparisons between each region*nitrogen treatment (significantly different letters indicated in red). In plot "C" CLD letter below the x-axis represent comparisons in belowground biomass, and error bars represent standard error. The dark red diamond within each boxplot represents the mean value, the upper and lower hinges of each boxplot represent the 25th and 75th quantile, the middle line represents the median value, the whiskers represent the 5th and 95th percentile, and outliers are represented by black points.

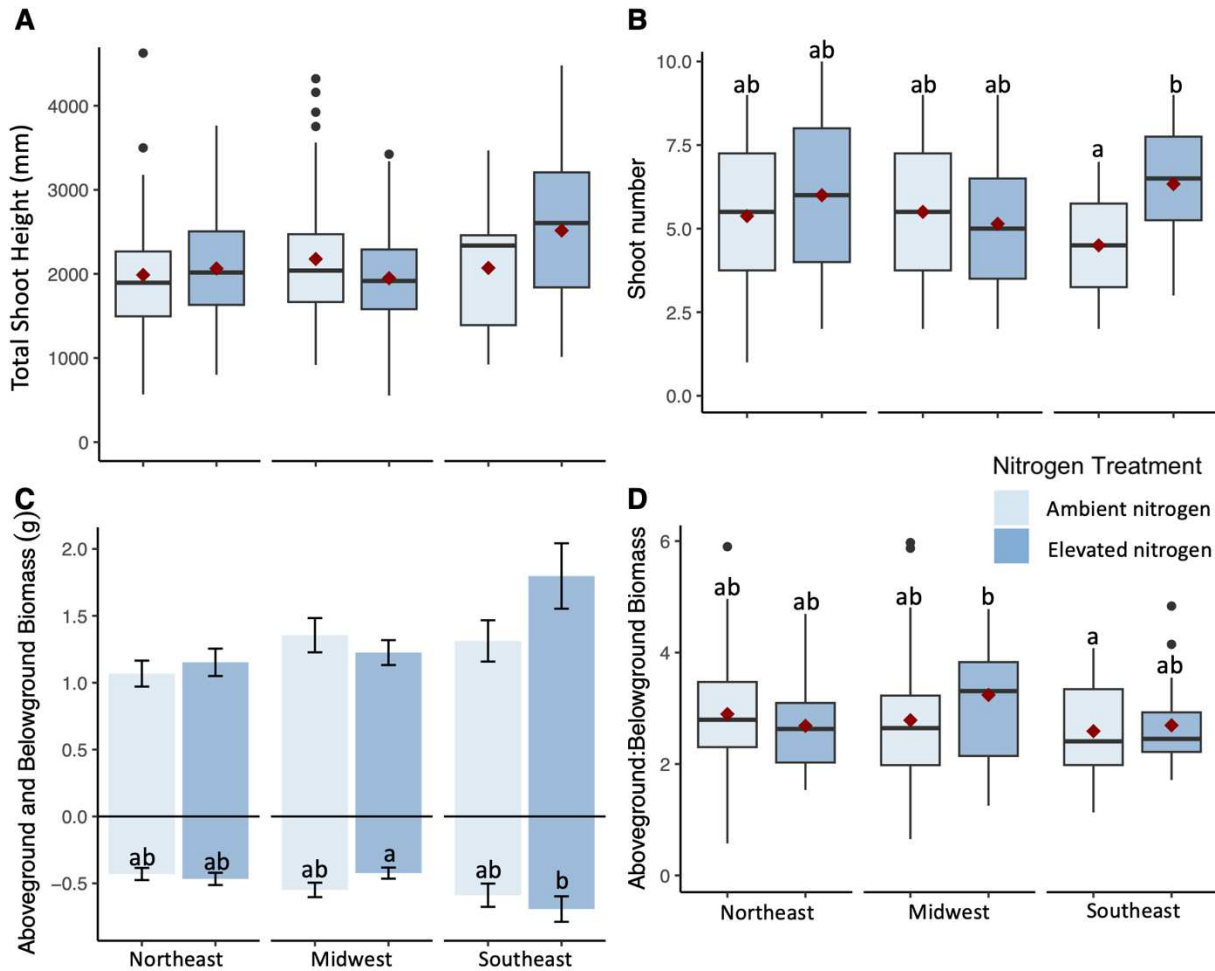
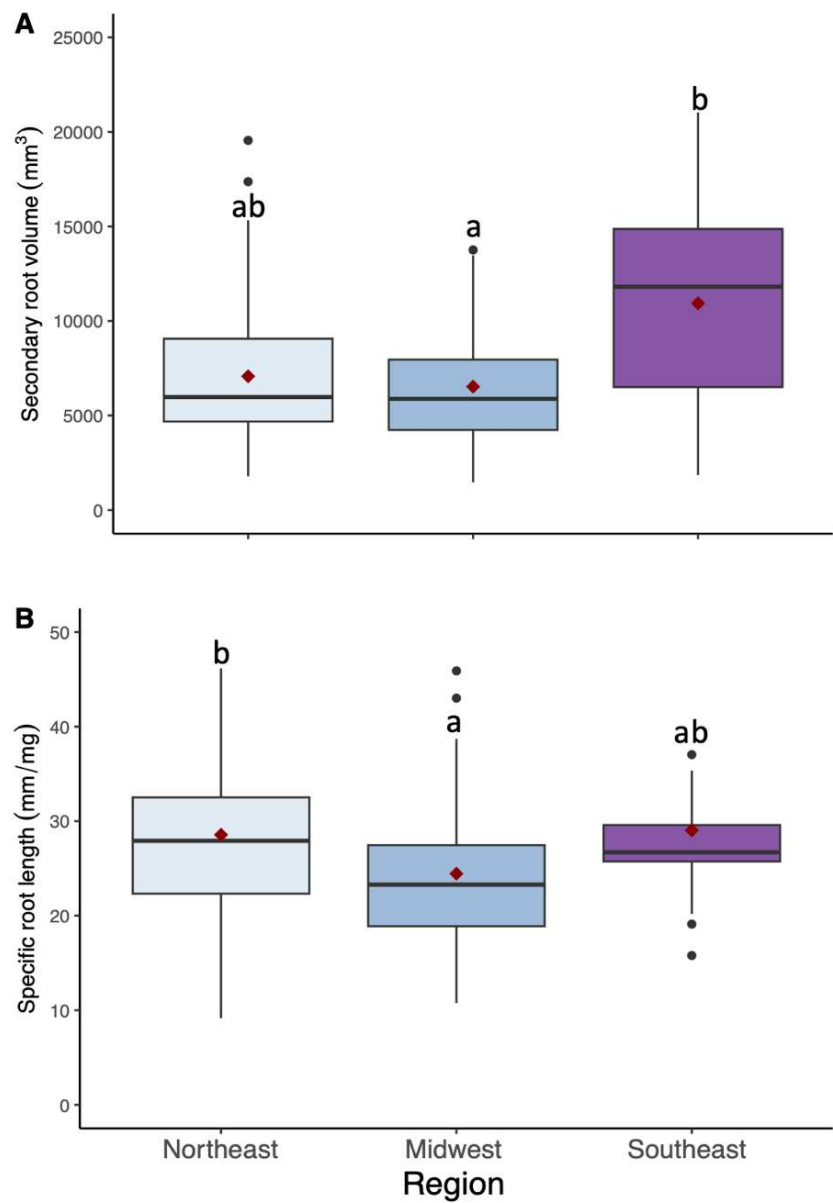


Figure 5 Mean of A) secondary root volume and B) specific root length by region of origin (Northeast, Midwest, and Southeast). Letters represent Tukey's HSD test displayed in compact letter design (CLD) showing significant pairwise comparisons between each region. The dark red diamond within each boxplot represents the mean value, the upper and lower hinges of each boxplot represent the 25th and 75th quantile, the middle line represents the median value, the whiskers represent the 5th and 95th percentile, and outliers are represented by black points. The box and whiskers in the violin plot represent the 10th quantile (lower whisker), 25th quartile (lower edge of box), midline (median), 75th quartile (top edge of box), and 90th quantile (top whisker).



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