SAMPLE PAPER

Iron nutrition of *Pinus elliotii* var. *densa* seedlings in two soils with different iron availability

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Abstract

Seedlings of endangered Dade County pine, *Pinus elliotii* var. *densa*, must survive long enough to form symbiotic associations with ectomycorrhizal fungi that assist uptake of mineral nutrients. Although plants need nitrogen (N), phosphorus (P), and potassium (K) in relatively large quantities, pine seedlings also may need relatively high amounts of iron (Fe). We hypothesized that iron would limit the growth of non-mycorrhizal pine seedlings in a low-Fe soil, but not in a soil with relatively abundant Fe. We grew pine seedlings in a three-factor, fully factorial experiment in pots in a greenhouse. The factors are two soils (flatwoods and sandhill) with different Fe availability from the Archbold Biological Station, three levels of weekly Fe addition (none; 37 mg; 74 mg chelated iron), and two levels of N, P, and K fertilization (none; one-time addition of 0.93, 0.28, and 1.85 g, N, P, K, respectively). At the final harvest, 18 weeks after treatments began, we measured chlorophyll *a* and *b* concentrations, dry weights of needles, stem, primary root, and fine roots. In the presence of NPK, iron had a negative effect on chlorophyll and biomass which might be the result of iron immobilizing phosphorus. In the absence of NPK, iron had a positive effect on chlorophyll and on root-to-shoot ratio which suggests that iron subtly limits seedlings in soils with little NPK, but on more fertile soils phosphorus might be the growth limiting nutrient.

Introduction

Adult pines and seedlings have ectomycorrhizae which are formed by symbiotic fungi in intimate association with roots and which facilitate the uptake of nutrients, especially inorganic and organic nitrogen (Read and Perez-Moreno, 2003). After germination, however, seedlings need to survive and take up nutrients on their own until they form ectomycorrhizae. In this experiment, we focus on pine seedling mineral nutrition during the period before ectomycorrhizae are formed.

Pinus elliotii var. *densa* (Dade county pine) is the only canopy species of pine rockland in South Florida, and the most abundant canopy tree of pine flatwoods and sandhill in Central Florida (Abrahamson et al, 1984; Abrahamson and Hartnett, 1990; Snyder et al, 1990). Natural fires are a key element for the maintenance of pine species (Wade et al, 1980). Low intensity fires will kill small shrubs of other species and leave the adult pines unharmed cleaning the ground for pine seedlings to emerge. On the other hand, a fire of high intensity threatens pine survival because it will consume the crown of adult pines resulting in total death (Menges and Deyrup, 2001).

Pine rockland ecosystem is seriously endangered in Dade County (South Florida) due to growing urbanization in this area. Because it is a keystone species of this ecosystem, *P. elliotii* var. *densa* has suffered an alarming decline in Florida during the last century (Snyder et al, 1990). The main causes for the disappearance of this species are disturbances in its habitat that interfere with the natural conditions necessary for its abundance. Fire regime alteration by humans is the main factor altering the pine rockland ecosystem. Preventing fires creates conditions for hardwood plants like oaks to take over pinelands (Snyder et al, 1990). In addition, suppression of frequent low intensity fires allows for the accumulation of large amounts of dry vegetation that serve as fuel for lethal high intensity fires.

Pine flatwoods of Central Florida face destruction due to fire suppression in areas near houses, and also because part of this ecosystem has been transformed into pastures for cattle and land for citrus crops. The Archbold Biological Station in Highlands County (Central Florida) is one of few places were flatwoods and sandhill ecosystems are not disturbed by human activity. At the station, flatwoods is found at low elevation while sandhill is at high elevations. Both ecosystems are dominated by Dade county pine over an understory of oaks, Saw palmetto (*Serenoa repens*), and Scrub palmetto (*Sabal etonia*) (Abrahamson et al, 1984).

Pine seedlings have been reported to be extremely scarce in Central and South Florida which raises concerns about the future of this pine species. Failure of the pine population to regenerate may result in its total disappearance in the next decades. The factors responsible for the low survival of seedlings are unknown, but shading of the seedlings by the bushes is suspected as well as iron deficiency. Indeed, an experiment conducted in South Florida demonstrated that iron has a positive effect on survival and synthesis of chlorophyll on *P. elliotii* var. *densa* seedlings grown in the field with and without ectomycorrhizae (David P. Janos, unpublished data).

In our experiment, we want to determine the effect of iron addition on survival, chlorophyll concentration and biomass in non-mycorrhizal *P. elliotii* var. *densa* seedlings grown in flatwoods and sandhill soil from Archbold Biological Station. In contrast to the circumneutral pH of South Florida rockland soil studied by Janos, Archbold Biological Station flatwoods and sandhill soils are very acidic. This difference in pH creates differences in iron availability (Sauchelli, 1969), iron being more accessible to plants in low pH soil. We used both flatwoods and sandhill soils because they differ in iron concentration. Sandhill soil contains more iron than flatwoods so we expect to see a bigger effect of iron addition to flatwoods soil. Because flatwoods and sandhill are relatively infertile soils, we added nitrogen, phosphorus, and potassium to half the seedlings in case a limitation of these three main nutrients would mask a response to iron addition.

Materials and Methods

Pine seedlings of *Pinus elliotii* var. *densa* were grown in a three-factor, fully factorial experiment in a green house. The factors were two soils (sandhill and flatwoods soil), three levels of iron (none, low, and high), and two levels of fertilizer (absent and present). The fertilizer contained nitrogen (N), phosphorus (P), and potassium (K).

The soils were collected at Archbold Biological Station located south of lake Placid in Central Florida 27°11’N lat., 81°21’W long. Both soils were sterilized to kill any microorganism and especially mycorrhizal fungi. All the soil was autoclaved three times for 60 minutes 121°C and 1.4 kg/cm^2 each time 24 hours apart.

Seeds of *Pinus elliotii* var. *densa* were provided by the Andrews Nursery (Florida Division of Forestry, Chiefland, FL, USA). Before planting, the seeds were soaked in water for 24 hours, and incubated at 4°C for 7 days. The seeds were germinated in coarse silica sand (L 6-20, Standard Sand and Silica Co., Miami, FL, USA) and were watered daily with abundant water. Approximately one month after planting the seeds, 96 seedlings of similar heights were randomly chosen and transplanted into 5x18 cm DeePots. Half of the DeePots contained flatwoods soils and the other half were filled with sandhill soil. On the day of the transplant, height, stem diameter, and needle length of all seedlings were measured. Using this initial data, the seedlings were randomly allocated to the iron and NPK treatments.

Chelated iron (Sequestrene 138, Becker Underwood, INC) was used in solution for the iron treatment. The high and low iron solution contained 7.4 mg/mL and 3.7 mg/mL of chelated iron respectively. The no-iron treatment consisted of distilled water. We added weekly 10 mL of each iron treatment three months after transplant. Florikan fertilizer was used in the form of slow release pellets. NPK treatments received a one-time addition of 0.93, 0.28, and 1.85 g, N, P, K, respectively beginning at the same time as the iron treatments.

All seedlings were given 20 ml of water twice a week after transplant. However, two months after starting the treatments, the initial watering regime appeared insufficient in the well-drained, sandy soils, so the frequency of watering was increased to four times a week.

Seedling Measurement

At the final harvest, needles, stem, primary root (tap root), and lateral roots were separated and needles were weighed fresh. A portion of about 0.2g of fresh needles were weighed and set aside for chlorophyll extraction. The remaining fresh needles were weighed again. The needles, stems, primary root and lateral roots were dried at 50°C for 3 days and the dry weight was measured. The total dry weight of needles was extrapolated from the dry weight of remaining needles.

For chlorophyll analysis the needles were incubated in 80% acetone for 48 hours in the dark (Proctor, 1981). The concentration of chlorophyll *a* and *b* were obtained by measuring absorbance of the supernatant at two wavelengths (663 and 645; Porra, 2002).

Statistical Analysis of Data

The effects of the different treatments on the chlorophyll concentrations and dry weights were analyzed as separate three-way ANOVAs with Statistix v. 8.0 software.

Results

We report soil differences between flatwoods and sandhill soils, and then report pine seedling responses to them and to fertilization. Seedling response variables include chlorophyll concentrations, dry weights of plant parts, root-to-shoot ratios, and seedling survival. There was never a three-way interaction among the soil, NPK, and iron treatments or a two way interaction between iron and soil (Table 1), but there were significant two-way interactions between soil and NPK and between NPK and iron, so our report reflects those particular two-way interactions.

Soil analysis

Sandhill soil is in general more fertile than flatwoods soil; sandhill is richer in iron and slightly more abundant in phosphorus. Flatwoods soil contains slightly more organic matter (which can be considered a surrogate for nitrogen) than sandhill soil and also flatwoods soil is more acidic (Table 2).

Chlorophyll

NPK fertilization had consistent, significant main effects on all three chlorophyll variables (chlorophyll *a*, chlorophyll *b*, and total chlorophyll; Table 1), and although iron had no significant main effects it interacted significantly with NPK for all three variables

(Table 1). NPK increased all chlorophyll variables, while iron had a tendency to increase chlorophyll in the absence of NPK, but high iron diminished chlorophyll in the presence of NPK (Figure 1).Soil only affected chlorophyll a (Table 1) by flatwoods soil having more chlorophyll a than sandhill soil (Figure 2), and soil interacted with NPK to affect chlorophyll b (Table 1). For chlorophyll b, although a conservative Tukey's HSD post hoc test did not support differences among means, the interaction was that without NPK, sandhill soil had more chlorophyll b than flatwoods soil, but when given NPK, seedlings in sandhill soil had less chlorophyll b than in flatwoods soil (Figure 2).

Plant dry weights

NPK addition had a significant strong positive effect on the dry weight of leaves, stems, primary roots, and total weight but not on fine root weight (Table 1, Figure 3). Iron had a significant main effect and interacted significantly with NPK on leaf weight and total weight (Table 1). Increases in iron concentration decreased leaf weight and total weight in the presence of NPK (Figure 3).Soil had a significant main effect on all variables (Table 1) by flatwoods plants being bigger than sandhill plants (Figure 4). Soil and NPK interacted significantly in all variables except for fine root weight (Table 1), with a stronger effect of NPK on flatwoods soil than in sandhill soil (Figure 4).

Root-to-shoot ratio

NPK significantly lowered the root-to-shoot ratio in plants (Table 1, Figure 5). NPK and iron interacted significantly to affect root-to-root ratio (Table 1). In the absence of NPK, iron decreased the root-to-shoot ratio while in the presence of NPK, iron tended to increase this ratio although this was not supported by Tukey's HSD post hoc test (Figure 5).

Survival

Survival was decreased by NPK as revealed at $P < 0.0001$by a two-tailed Fisher Exact Test (Figure 6a). Neither soil (Figure 6b) nor iron (Figure 6c) affected survival (two-tailed Fisher Exact Test $P = 0.238$ and Chi-square = 2.33, $P = 0.311$ for soil and iron, respectively).

Discussion

NPK addition favored synthesis of both chlorophyll *a* and *b* as expected. Chlorophyll *a* is found in larger amounts than chlorophyll *b*. Chlorophyll *a* is the primary photosynthetic pigment while chlorophyll *b* is an accessory pigment that expands the photosynthetic wavelength range. The positive effect of iron on chlorophyll *a* and *b* was only seen in the absence of NPK, probably because the strong positive effect of NPK hides the positive effect of iron (Figure 1). This indicates that iron could be of benefit to pine seedlings growing in soils relatively scarce in nutrients such as flatwoods and sandhill soil. The negative effect of iron on chlorophyll concentration observed in plants with NPK could be the result of the iron immobilizing phosphorous from the fertilizer and therefore making it less available to the plant. In contrast, seedlings in sandhill soil probably had a lower concentration of chlorophyll *a* than those in flatwoods soil (Figure 2) because of lower nitrogen availability in sandhill than in flatwoods soil which may be inferred from percentage organic matter (Table 2). That seedlings in sandhill soil contained more chlorophyll *b* than flatwoods seedlings in the absence of NPK does not make a difference in total plant chlorophyll (Figure2). In addition, the concentration of chlorophyll *b* is not as crucial to the plant as the concentration of chlorophyll *a*.

NPK notably increased needles, stem, and primary root dry weights, but it did not make a difference in fine root weights (Figure 3). Seedlings with NPK were provided with their most required nutrients so they were able to increase substantially in shoot. However, seedlings with fertilizer do not need to expend energy and resources on expanding their roots to find deeper or more distant nutrients; this explains why NPK did not affect fine root weight. The growth facilitated by NPK all was allocated aboveground. The clear decreases in leaf weight attributable to iron in the presence of NPK (Figure 3) correlates with the negative effect of iron on chlorophyll concentration in plants with NPK, and this could also be the result of phosphorus sequestration by iron.

Dry weight differences between seedlings in flatwoods and sandhill soil were consistent with the results of the chlorophyll analysis. Dry weights of flatwoods plants with and without NPK were higher than dry weights of sandhill plants with and without NPK (Figure 4) which most likely reflects the inferred higher availability of nitrogen in flatwoods soil than in sandhill soil.

Root-to-shoot ratio was lower in seedlings with NPK (Figure 5) because of the shift of allocation aboveground, especially to needles. With increased mineral nutrient availability, a relatively small proportion of fine roots could sustain a large shoot biomass. Iron increased root-to-shoot ratio in the presence of NPK (Figure 5) which suggests that iron made seedlings grow more roots probably because they were being limited by phosphorus. When NPK was absent, Iron seemed to have had a positive effect lowering the root-to-shoot ratio (Figure 5).

We attribute the surprising negative effect of NPK on survival to the difference in water demand of large seedlings with NPK versus that of small seedlings without NPK. Twenty-four of the 96 seedlings used in this experiment died, all of which had been provided with NPK. Seedlings with NPK developed more or bigger needles than those without NPK, and therefore they were in the need of more water than plants without NPK. Because we gave the same amount water to all seedlings in the different treatments, it is possible that seedlings with NPK did not receive enough water to support their positive response to NPK fertilization. Bengston (1976) somewhat similarly found that nitrogen and phosphorus reduced survival of *Pinus elliotii* var. *elliotii* 1 to 5 years-old saplings planted in the field on sandhill soil, but he attributed that to an extreme nutrient imbalance caused by nitrogen addition in the absence of potassium, magnesium, and sulfur addition. We added nitrogen, phosphorus, and potassium simultaneously, which in Bengston's experiment negated the detrimental effects of nitrogen and phosphorus addition without potassium.

Conclusions

There were no significant differences in response to iron between the two types of soil. In the presence of NPK, iron had a negative effect on chlorophyll, biomass and root-to-shoot ratio attributed to phosphorus immobilization. In the absence of NPK, iron had a positive effect on chlorophyll and on root-to-shoot ratio. Iron seems to be a limiting nutrient of pine seedlings when these are growing on soil with very small concentrations of nitrogen, phosphorus and potassium, but in soils rich in these nutrients, iron may have a negative effect on phosphorus acquisition by seedlings.

Acknowledgments

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Table 1
Results of statistical analyses. The top number in each cell is the *F* value obtained by 3 way ANOVA and the bottom number is the simple probability value (*P*) for that *F*.

	DF	Chlorophyll A	Chlorophyll B	Total chlorophyll	Leaf wt.	Stem wt.	Primary root wt.	Fine root wt	Total plant wt.	Root to shoot ratio
Soil	1	4.71	0.46	3.45	26.27	17.36	9.20	25.54	35.85	0.75
		0.0339	0.5002	0.0681	**0.0000**	**0.0001**	**0.0036**	**0.0000**	**0.0000**	0.3910
NPK	1	241.49	90.19	209.45	220.98	64.58	24.40	0.87	163.57	255.15
		<0.0001	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.3555	**<0.0001**	**<0.0001**
Iron	2	1.07	1.00	1.12	5.86	0.73	0.35	0.85	4.83	0.59
		0.3491	0.3743	0.3342	**0.0074**	0.4840	0.7075	0.4322	**0.0114**	0.5592
Soil x	1	0.27	5.52	1.00	9.52	8.17	5.35	0.64	10.34	0.71
NPK		0.6034	**0.0222**	0.3220	**0.0031**	**0.0058**	**0.0242**	0.4269	**0.0021**	0.4025
Soil x	2	1.38	1.44	1.30	1.66	1.23	0.53	0.88	1.96	1.48
Iron		0.2582	0.2452	0.2805	0.1986	0.2985	0.5904	0.4207	0.1491	0.2348
NPK	2	3.75	6.17	4.51	7.37	0.30	0.59	0.04	4.56	3.74
x Iron		**0.0293**	**0.0037**	**0.0150**	**0.0014**	0.7404	0.5567	0.9645	**0.0144**	**0.0295**
Soil x	2	0.41	0.11	0.32	2.82	0.86	0.83	0.39	2.18	2.15
NPK		0.6654	0.8919	0.7270	0.0673	0.4270	0.4408	0.6762	0.1216	0.1255
x Iron										
Error	60									
Total	71									

Table 2
Nutrients and properties of flatwoods and sandhill soils. OM = organic matter; P1 and P2 = Bray 1 and Bray 2 extractions, respectively; CEC = cation exchange capacity.

	pH	OM %	P1 mg/kg	P2 mg/kg	K mg/kg	Mg mg/kg	Ca mg/kg	Zn mg/kg	Mn mg/kg	Fe mg/kg	Cu mg/kg	CEC Meq/100 g
Flatwoods	**3.5**	**1.6**	**1**	**2**	**2**	**3**	**8**	**0.1**	**1**	**11**	**0.1**	**0.1**
Sandhill	**4.5**	**1**	**2**	**4**	**2**	**2**	**12**	**0.2**	**1**	**21**	**0.1**	**1.4**

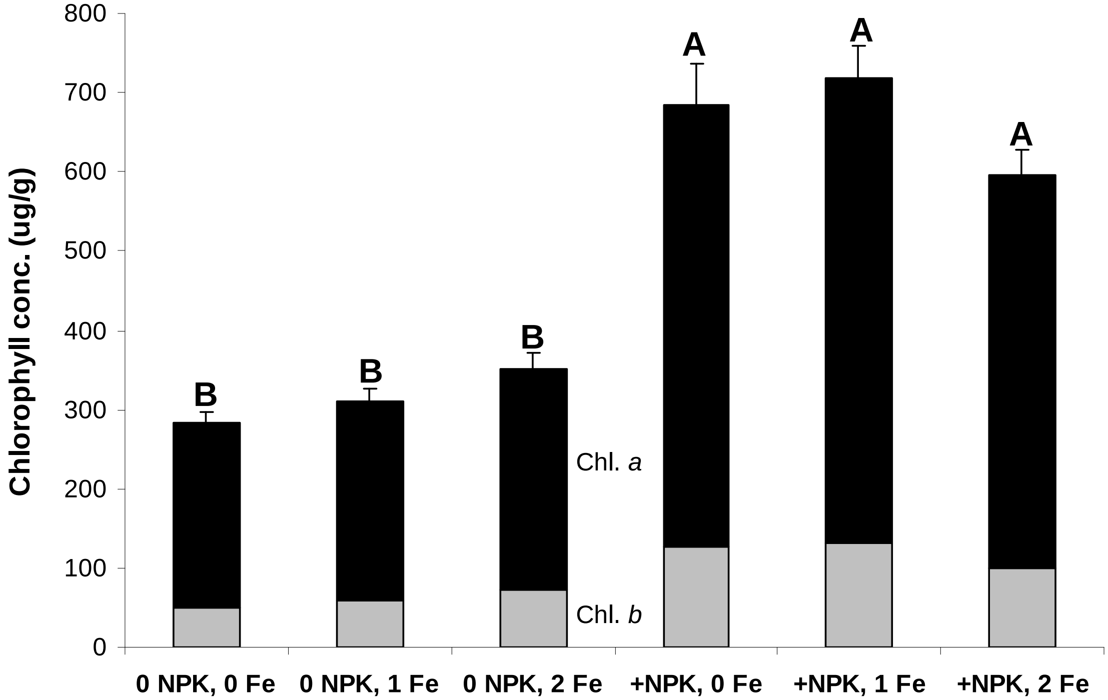

Figure 1. Mean chlorophyll *a* and *b* concentration (± 1 SE) in needles of seedlings with or without NPK with different levels of iron addition (no addition = 0 Fe; low concentration [37 mg Fe added weekly] = 1 Fe; and high concentration [74 mg Fe added weekly] = 2 Fe).

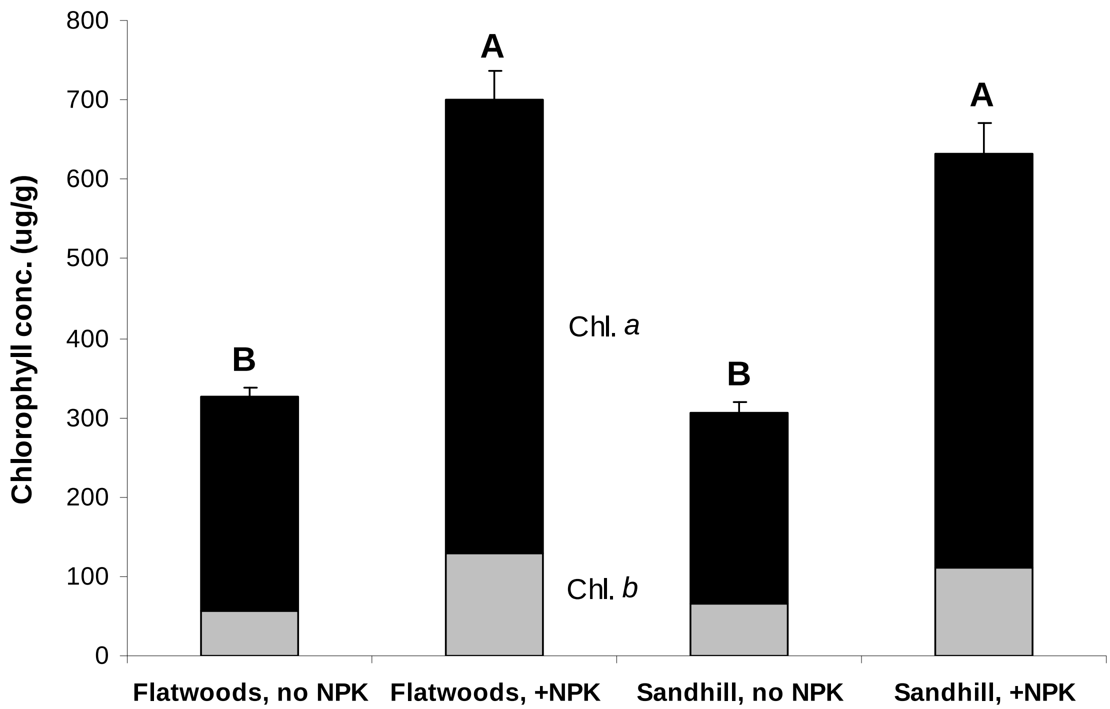

Figure 2. Mean Chlorophyll *a* and *b* concentration (± 1 S E) in pine needles growing either in flatwoods or sandhill soil, with or without NPK addition.

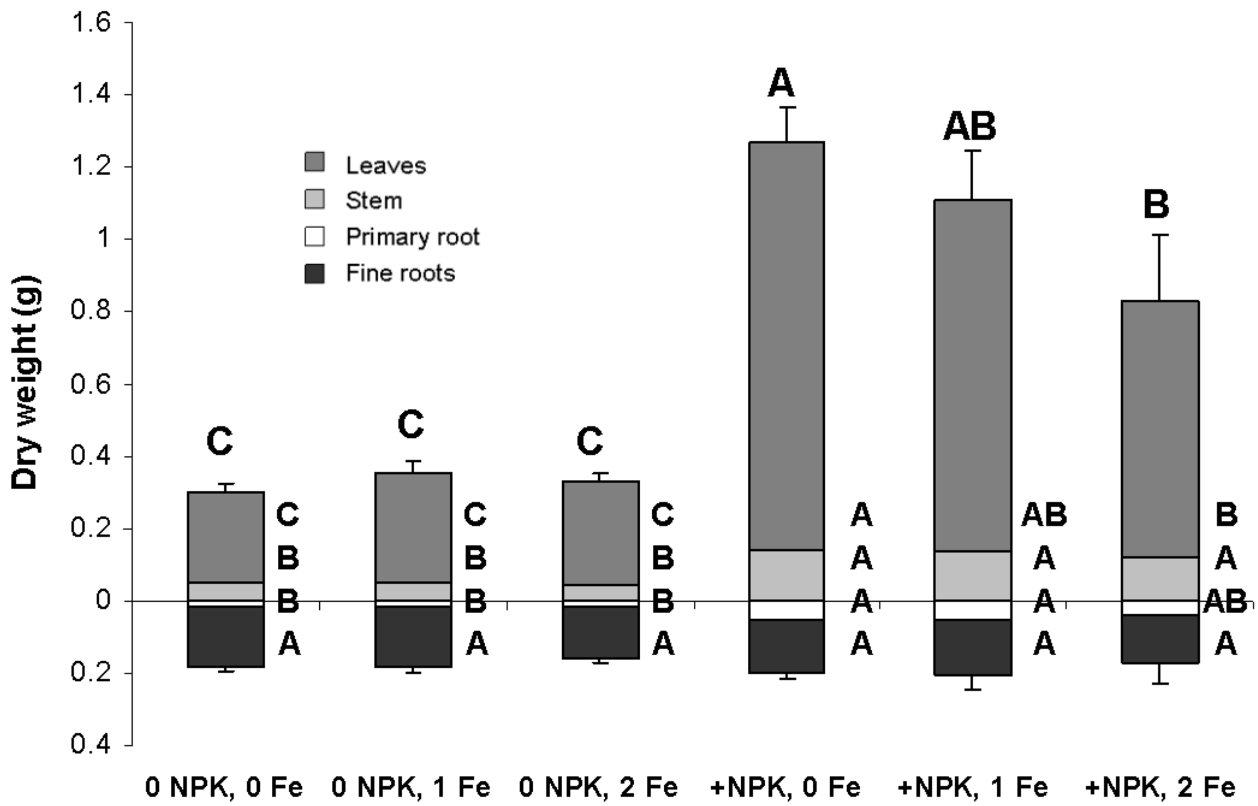

Figure 3. Mean dry weights of leaves, stems, primary roots and fine roots of seedlings (± 1 S E) with and without NPK with different levels of iron addition (no addition = 0 Fe; low concentration [37 mg Fe added weekly] = 1 Fe; and high concentration [74 mg Fe added weekly] = 2 Fe).The X-axis represents ground level; above it are the shoot variables (leaves and stem), and below the axis are the root variables (primary root and fine roots) shown as positive values.

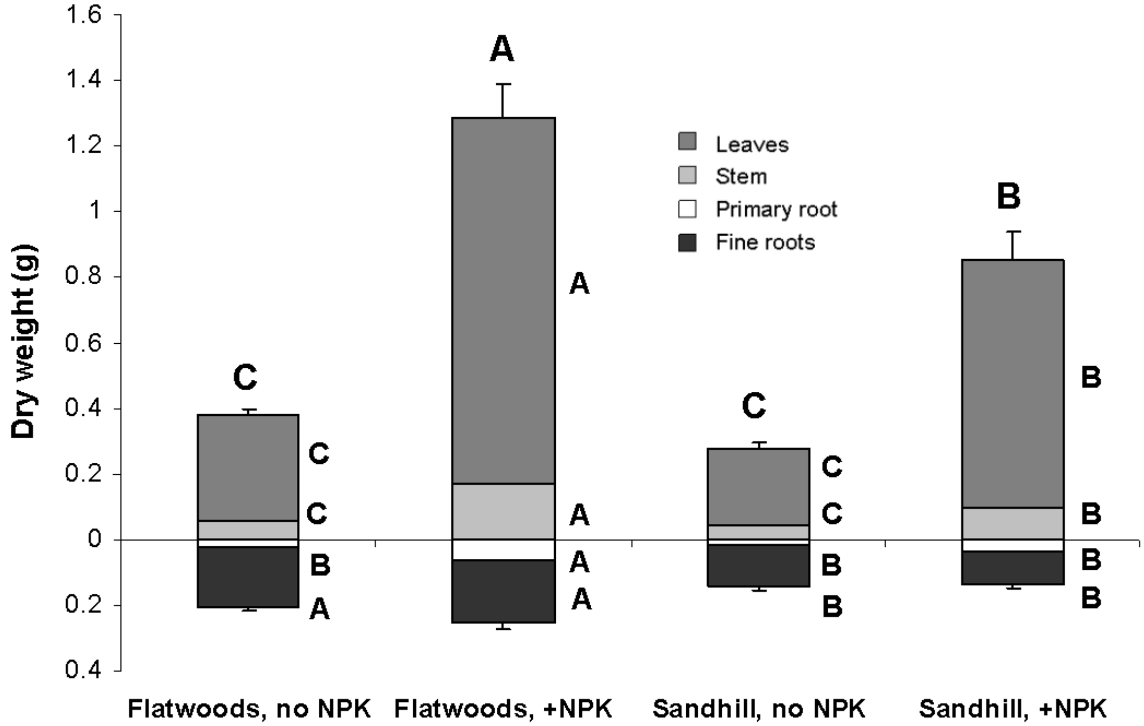

Figure 4. . Mean dry weights of leaves, stems, primary roots and fine roots of seedlings (± 1 S E) grown in either sandhill or flatwoods soil with and without NPK. The X-axis represents ground level; above it are the shoot variables (leaves and stem), and below the axis are the root variables (primary root and fine roots) shown as positive values.

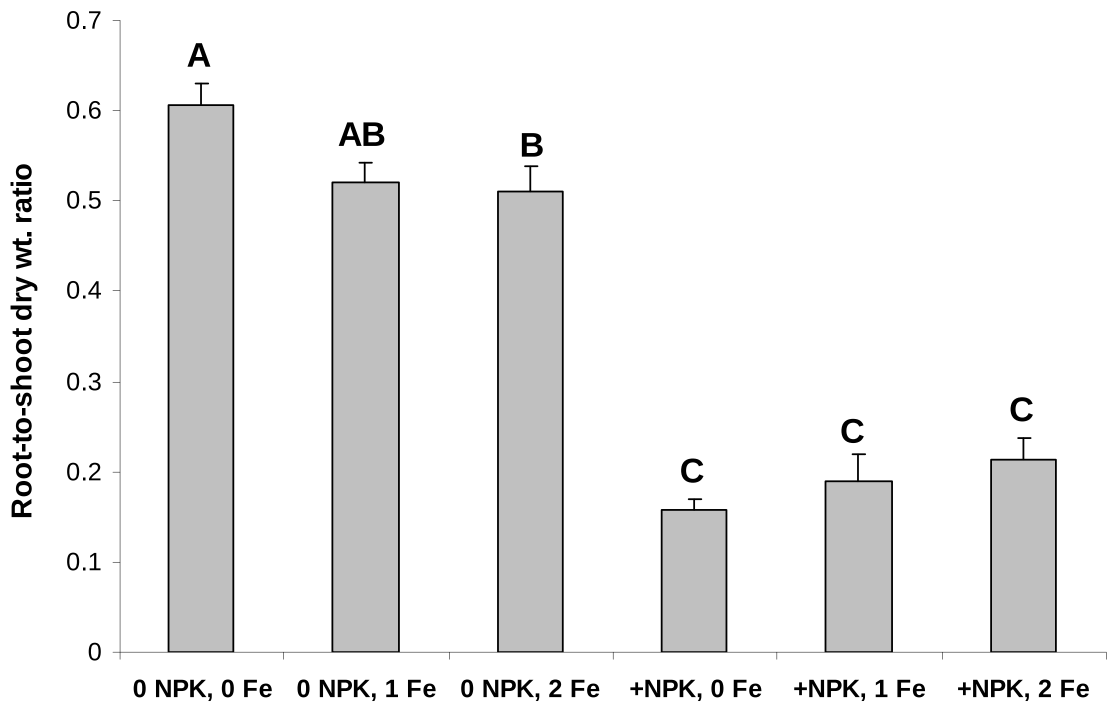

Figure 5. Mean root-to-shoot ratio of seedlings (± 1 S E) with and without NPK with different levels of iron addition (no addition = 0 Fe; low concentration [37 mg Fe added weekly] = 1 Fe; and high concentration [74 mg Fe added weekly] = 2 Fe).

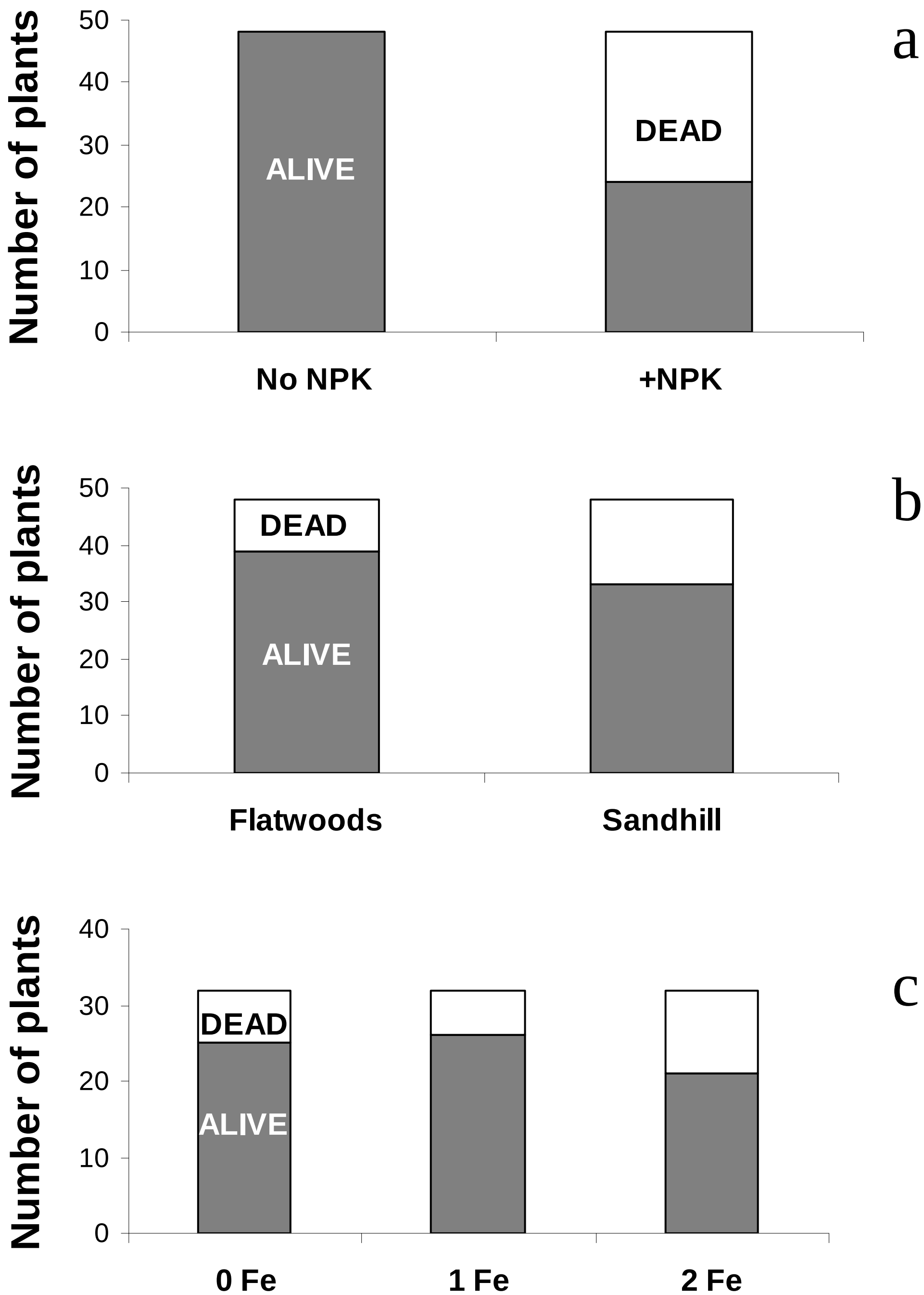

Figure 6. Number of surviving seedlings a) with and without NPK, b) in flatwoods versus sandhill soil, and c) at three levels of iron addition.