Effects of nitrogen and phosphorus availability on the early growth of two congeneric pairs of savanna and forest species

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Abstract
In the tropical region, savannas and seasonal forests, both highly diverse biomes, occur side by side, under the same climate. If so, that mosaic cannot be explained solely by climatic variables, but also by fire, water availability and soil status. Nutrient availability in the soil, especially nitrogen and phosphorus, has been postulated to explain the abrupt transitions between savannas and seasonal forests in tropical regions. Plants from these two biomes may present different nutritional strategies to cope with nitrogen and phosphorus limitation. We used two congeneric pairs of trees — each pair with a species from the savanna and another from the neighboring seasonal forest — to test whether savanna and forest species presented different nutritional strategies during their early development. We cultivated 56 individuals from each of these species in a hydroponics system with four treatments: (1) complete Hoagland solution, (2) Hoagland solution without nitrogen, (3) Hoagland solution without phosphorus, and (4) Hoagland solution without nitrogen and phosphorus. After 45 days, we harvested the plants and measured total biomass, root to shoot ratio, height, leaf area, and specific leaf area. Overall, savanna species were lighter, shorter, with smaller leaves, higher specific leaf areas, and higher root to shoot ratios when compared to the forest species. Nitrogen increased the performance of species from both biomes. Phosphorus improved the performance of the forest species and caused toxicity symptoms in the savanna species. Hence, savanna and forest species presented different demands and were partially distinct already as seedlings concerning their nutritional strategies.

Keywords: forest, hydroponics, nitrogen, phosphorus, savanna.

1. Introduction
Savannas are characterized by the presence of grasses, sub-shrubs, shrubs, and trees, in varying proportions, and temporal patterns related to seasonality and fire (Maurin et al., 2014). That biome is typical of the tropical areas, which harbor high biodiversity and some of the most threatened biomes, including the savannas and forests,
that often appear in mosaic (Beerling and Osborne, 2006; Kinlock et al., 2017; Myers et al., 2000). The occurrence of savannas and their physiognomic gradient are related not only to climate and fire, but also to herbivory, water availability, carbon dioxide, and soil nutrients (Beerling and Osborne, 2006; Oliveras and Malhi, 2016; Pinheiro and Monteiro, 2010).

Border dynamics between savannas and forests remain poorly understood, despite being intensively studied (Murphy and Bowman, 2012). However, savanna and forest species belong to different functional groups (Silva et al., 2013) and may present different strategies that enable them to respond differently to environmental factors (Barbosa et al., 2014; Miatto et al., 2016). For instance, whereas savanna species tend to be water and nutrient-limited, forest species tend to be limited by light (Dantas et al., 2015). Thus, climate change, soil eutrophication, soil impoverishment, and fire regime may affect the border dynamic between both physiognomies and favor one biome over the other (Hill and Southworth, 2016).

Soils from these two adjoining biomes are very different (Cruz Ruggiero et al., 2002). Savanna soils are usually older and sandy, whereas forest soils are generally younger and clayey (Cruz Ruggiero et al., 2002). Consequently, they present different leaching patterns and nutrient availability, especially concerning nitrogen and phosphorus (Tahir and Marschner, 2017). In savannas, the more intense leaching, the strength of ion adsorption, the frequent fires that volatilize some nutrients, and the older age make the soil more acid, richer in Al⁺⁺ and poorer in nitrogen and phosphorus than in forests (Pinheiro and Monteiro, 2010; Vitousek et al., 2010). Since these features can intensely change over short distances, it allows a mosaic in the spatial distribution of these two biomes (Souza and Martins, 2004).

Nitrogen and phosphorus are strong environmental filters, regulating the establishment and prevalence of either savannas or forests, mainly in neighboring areas (Almeida et al., 2018; Pellegrini, 2016). As long as their cycles are being intensively modified, it may pose new challenges to plants (Peng et al., 2019). Their availabilities can also change the savanna and forest functional attributes and trade-offs in biomass allocation differently (Poorter et al., 2012; Zhang et al., 2018). For example, functional traits required for light captures such as height and leaves can be higher in forest species; the trade-off for savanna species, in turn, can be shifted to belowground resources, by high roots investment (Paganeli et al., 2020). Since savanna and forest species evolved under different environmental scenarios, nutritional conditions may impose distinct pressures for the species of these two biomes in their early development (Hoffmann and Franco, 2008; Poorter et al., 2012).

Studies that aim to understand the effects of different nutritional conditions on species strategies may be useful to predict the dynamics of savanna-forest borders in future scenarios (Viani et al., 2011). In this sense, we tested the effects of different nutritional conditions regarding nitrogen and phosphorus availability on two congeneric pairs of savanna and forest species. Thus, we tried to answer the following questions: (1): Do forest plants have more biomass than the savanna plants? (2): Is the root-to-shoot ratio higher in savanna species than the forest ones? (3): Are the forest species taller than the savanna ones? (4): Do the forest species present larger leaves and specific leaves areas than savanna species? (5): Are biomass, height, and leaf area higher when both, savanna and forest seedlings grow on the complete solution?

2. Materials and Methods

To account for phylogenetic relatedness (Hoffmann and Franco, 2008), we used two congeneric pairs of species, each pair with one species from the savanna and another from the neighboring seasonal forest. The first pair belonged to the genus Solanum L. (Solanaceae), with S. lycocarpum St. Hil. from the savanna and S. grandiflorum Ruiz and Pav. from the forest; the second pair belonged to the genus Enterolobium Mart. (Fabaceae), with E. gunneriflorum (Mart.) J. F. Macbr. from the savanna and E. contortisiliquum (Vell.) Morong from the forest. All species are common in central Brazil, in their vegetation types.

We purchased the seeds from nurseries and germinated them in the laboratory. We moistened germitest paper sheets with 10 ml of distilled water (Lessa et al., 2015). We mechanically scarified the Enterolobium seeds and placed 20 of them on Petri dishes with two germitest paper sheets above the seeds and two below. We wrapped the dishes with plastic film and took them to an incubator at 25 °C and 12-hour photoperiod (Malavasi and Malavasi, 2004). In the Solanum species, we used 12 hours alternating light and dark and high and low temperatures – 30 and 20 °C (Pinto et al., 2007). By using germitest paper sheets as substrate, we reached low germination rates. Thus, adjustments were performed using an alternative inert substrate, which displayed good germination rates, the expanded clay.

We sieved the clay with a 2 mm soil sieve. Then we sieved the content again with a 1 mm sieve. Thus, we standardized the grain size between 1 and 2 mm. We washed the substrate repeatedly and abundantly with running water in the first times and with distilled water in the last. We also sterilized it in an ultraviolet chamber for 15 minutes. On each dish, we put 55 g of clay, 20 seeds on top of this layer, and finally put 55 g of clay. We moistened each dish with 45 mL of distilled water. When the radicles were 1 cm long, we randomly picked 56 seedlings of each species and placed each seedling in a 180 cm³ plastic tube, filled with expanded clay previously described.

During the 2019 summer, we took the plastic tubes to a greenhouse located in São Carlos (21°59'S, 47°53'W; southeastern Brazil), where cooling, humidification, ventilation, and exhaustion were automatically controlled, so that the temperature was kept between 20°C and 28°C, the air relative humidity between 60% and 80% under a natural light regime. To test the effect of nitrogen and phosphorous depletion on plant growth, we cultivated the seedlings in closed hydroponic systems, in which the nutrient solutions were recycled (Prado and Casali, 2006; Jensen, 1997). We used four treatments: (1) complete Hoagland solution, (2) Hoagland solution without nitrogen, (3) Hoagland solution without phosphorus, and
In the cells, there are the volumes (ml) of different 1M stock solutions previously prepared and added to 90 L of distilled water. The solutions were returned to the reservoir by gravity.

For each congeneric pair, we placed eight boxes on aluminum stands. For each treatment, we used two boxes, one for the savanna species and the other for the forest species. To prevent algae proliferation, we covered the boxes with aluminized thermal blankets to avoid light in the solutions. We filled each box with 40 L of its respective solution. Then, we placed support for 14 plastic tubes. The two boxes of each treatment were connected to a 100 L reservoir by a silicone hose, at one end with a T-connector, and at the other end with a submersible motor pump that remained inside the reservoir. The solution was propelled by the motor pump, going through the hose, to the T-connector, and the two boxes. We set a timer to propel the solution in five cycles daily: at 06:30, 10:30, 12:00, 14:30, and 17:30. The timer remained on for 3 min, the time necessary to move the solutions, homogenize them, and moisten the clay. At the end of each cycle, the solution returned to the reservoir by gravity.

After 45 days, we harvested 14 individuals of each species and each treatment, oven-dried them at 80 °C for 72 hours, and measured total dry biomass. Biomass is an important functional trait since it is considered the best predictor of community functioning and plant performance (Gignoux et al., 2016; Grime, 1998). We also separated the below from the aboveground portion and weighed their dry masses. Dividing the below by the aboveground part, we obtained the root-to-shoot ratio, which is appropriate to assess biomass allocation (Poorter et al., 2012). Plants adapted in oligotrophic conditions are expected to present higher biomass allocation to roots so that the ratio is a good indicator of resource limitation (Poorter et al., 2012). We measured height by taking the distance from the hypocotyl to the apical bud. We scanned the leaves and measured their areas. We divided the leaf area by dry leaf mass to calculate the specific leaf area. Height and leaf traits as specific leaf areas play an important role in plant development by improving the light interception, serving as a proxy of its growth performance (Rodriguez and Maiti, 2016).

To answer the five questions posed, we used covariance analyses. In a given model, the response variable was total dry biomass, root to shoot ratio, height, leaf area or specific leaf area; the explanatory variables were biome (savanna or forest), nitrogen (presence or absence), and phosphorus (presence or absence). We also included the interaction terms as explanatory variables (nitrogen + phosphorus, nitrogen + biome, phosphorus + biome, and nitrogen + phosphorus + biome). In the case of the specific leaf area, we also tested whether it was related to height, root-to-shoot ratio, and biome.

### Table 1

<table>
<thead>
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<th>Treatments</th>
<th>KH₂PO₄</th>
<th>KNO₃</th>
<th>Ca(NO₃)₂</th>
<th>MgSO₄</th>
<th>KCl</th>
<th>CaCl₂</th>
<th>Micronutrients</th>
<th>Ferric and monosodium EDTA</th>
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<td>-P</td>
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<td>90</td>
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<tr>
<td>-NP</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>180</td>
<td>135</td>
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3. Results

Total biomass was related to the explanatory variables considered together (R² = 0.40; P < 0.001), particularly to nitrogen (P < 0.001), phosphorus (P = 0.005), biome (P < 0.001), the interaction term between nitrogen and biome (P < 0.001), and the interaction term between nitrogen and phosphorus (P = 0.003). Individuals of forest species (1.73 g ± 1.59, mean ± sd) displayed heavier dry total biomasses (P < 0.001) than individuals of savanna species (0.66 g ± 0.77). Savanna species were heavier in the complete solution (1.27 g ± 0.80) than without phosphorus (0.67 g ± 0.95). Interestingly, the solution lacking both macronutrients had higher total biomass (0.42 g ± 0.40) than that missing nitrogen and phosphorus (0.42 g ± 0.40), and that missing nitrogen (0.28 g ± 0.34). Forest species were heavier in the complete solution (3.05 g ± 1.91) than without phosphorus (1.99 g ± 1.36), nitrogen (0.98 g ± 1.02), and both (0.90 g ± 0.84).

Root to shoot ratio was related to the explanatory variables considered together (R² = 0.47; P < 0.001), higher (P < 0.001) in savanna (0.68 ± 0.30) than in forest species (0.38 ± 0.12). Both nitrogen (P < 0.001) and phosphorus (P < 0.001) affected this trait significantly. Savanna species presented higher ratio in the solution without both nutrients (0.90 ± 0.16), than in the complete solution (0.48 ± 0.17), without nitrogen only (0.40 ± 0.73), and without phosphorus only (0.25 ± 0.59). Forest species presented higher ratio in the solution without phosphorus only (0.42 ± 0.09), then without both nutrients (0.39 ± 0.12), without nitrogen only (0.38 ± 0.13), and in the complete
solution (0.30 ± 0.09). The interaction term between nitrogen and biome was also significant (P < 0.001) (see Figure 1).

Individuals of the forest (23.14 cm ± 18.6) were taller (R² = 0.28; P < 0.001) than those of the savanna (11.25 cm ± 6.4). Height was related to nitrogen (P < 0.001), but not to phosphorus (P = 0.154). The effect of nitrogen on height depended on the biome (P = 0.001). Individuals of the savanna species grew taller in the complete solution (14.48 ± 6.15), then without phosphorus only (11.12 ± 6.04), without nitrogen (9.92 ± 6.68), and without both nutrients (9.5 ± 5.81). Individuals of the forest species grew taller in the complete solution (33.53 ± 21.71), then without phosphorus only (27.05 ± 20.16), without both nutrients (16.15 ± 12.49), and nitrogen only (15.82 ± 12.98) (see Figure 2).

Leaves were larger (R² = 0.75; P < 0.001) in forest (201.40 cm² ± 157.16) than in savanna species (72.90 cm² ± 58.23). All explanatory variables were significant (P < 0.01 in all cases). Savanna species presented larger leaves in the complete solution (142.34 ± 29.15), then without phosphorus only (67.09 ± 54.75), without nitrogen (43.95 ± 42.35), and without both nutrients (37.74 ± 32.25). Forest species presented larger leaves in the complete solution (399.91 ± 95.59), then without phosphorus only (225.30 ± 107.14), without nitrogen only (90.92 ± 91.09), and both nutrients (89.45 ± 73.75).

Specific leaf area was not related to the explanatory variables taken all together (R² = 0.05; P = 0.113), but it was marginally different between the biomes (P = 0.055), higher in savanna (552.06 cm² g⁻¹ ± 1050.62) than in forest species (360.32 cm² g⁻¹ ± 198.16). The interaction terms between nitrogen and phosphorus (P = 0.069) and among nitrogen, phosphorus, and biome (P = 0.046) were, respectively, marginally significant and significant. Savanna species presented higher specific leaf areas in the solution without nitrogen (791.12 cm² g⁻¹ ± 1560.89), then in the solution without phosphorus (695.36 cm² g⁻¹ ± 1285.83), in the complete solution (416.03 cm² g⁻¹ ± 505.46), in the solution without both nutrients (300.60 cm² g⁻¹ ± 179.51). Forest species presented higher specific leaf areas in the solution without nitrogen (393.6 cm² g⁻¹ ± 245.36), then in the complete solution (372.50 cm² g⁻¹ ± 202.14), in the solution without both nutrients (358.88 cm² g⁻¹ ± 217.64), and the solution without phosphorus (302.30 cm² g⁻¹ ± 108.29). Specific leaf area was related to height, root to shoot ratio, and biome (R² = 0.35; P < 0.001).

4. Discussion

Concerning their growth under different nitrogen and phosphorus supply, congeneric savanna and forest species displayed distinct functional traits and patterns of biomass increment and allocation from the very early stages of their life cycles, as predicted, for example, by Poorter et al. (2012) and Zhang et al. (2018). Although species from these biomes live under different nutrient availability (Cruz Ruggiero et al., 2002), both congeneric pairs that we analyzed were limited by nitrogen. Overall, when they were supplied with that element, they grew faster and higher. Phosphorus, on its turn, increased the performance, but in general, the growth improvement was less pronounced than those provided by the nitrogen.

Such pattern was evident, for instance, in total biomass, with species from the two biomes showing different responses to nutrient addition. Biomass increment provided by nitrogen was enhanced by phosphorus in species from both biomes. However, in the savanna species, when there was no nitrogen, the addition of phosphorus reduced the biomass acquisition — perhaps an indication of phosphorus toxicity (Mohidin et al., 2015; Pagani et al., 2020; Silber et al., 2002). (see Figure 2). As phosphorus availability in savannas tends to be low, species from this biome may present strategies to cope with such hindrances, as low demand and growth rates (Matzek and Vitousek, 2009; Pellegrini 2016). Since the seedlings in general usually present a high mortality rate (Collet and Le Moguedec, 2007), if it decreases the external phosphorus consume in the early stages of life, this strategy reduces the phosphorus competition at the community level, even if momentary (Pagani et al., 2020). On the other hand, the forest species displayed high nutritional demand, shown by intense biomass reduction when growing in solutions with nitrogen or phosphorus absence.

These growth patterns could be fit into different plant nutritional strategies, the conservative (adopted by less demanding species) and the acquisitive ones (typical for fast-growing species), which allow savanna and forest species to live in contrasting habitats in neighboring sites (Caplan et al., 2017; Chacón-Madrigal et al., 2018; Maracahipes et al., 2018). Both strategies are consistent with our results and the evolutionary history of savanna and forest species. It makes possible the prevalence of one vegetation type over the other concerning competition

Figure 1. On that figure are the logarithms of root-to-shoot ratio square root in two pairs of congeneric species. The forest houses data from two species (E. contortisiliquum and S. grandiflorum), as do the savanna (E. gummiferum and S. lyocarpum). The complete solution is represented by Com; The solution without N by -N; The solution without P by -P; The solution without both nutrients by -NP.
Nutritional strategies in savanna and forest congeneric species under the current and the future nutrients unbalanced inputs, changing the functional diversity on those mosaics (Paganeli et al., 2020; Peng et al., 2019; Wallis and Bobbink, 2017).

Admittedly, environmental quality and community functioning are complex and cannot be defined solely by total biomass (Norberg, 2004). Regarding plant biomass, the root to shoot ratio can be interpreted as where — below or aboveground — the incorporated organic matter was preferentially allocated, allowing species to be more adapted to forage with the roots or the aerial organs (Tuller et al., 2018). It also implies differences in vegetation structure (Potter and Klooster, 1997) and, consequently, provides information about ecological interaction below or aboveground (Schuldt et al., 2017).

Since the savanna species evolved in poorer soils, this process could be visualized by the strategy of higher root to shoot ratio in savanna species when compared to forest ones (Miatto et al., 2016; Qi et al., 2019). The savannas species displayed a huge ability to forage for nutrients, and more intensive capacity related to nitrogen. When those species grew in solution without this element, the root biomass allocation almost doubled, and the forest species showed a weak mechanism to nutrient foraging since its trait does not change according to treatments as the savanna species did. It exemplifies different evolutionary processes in these two neighboring biomes, which could have culminated in niche differentiation and speciation, diminishing competition, and favoring their coexistence (Rueffler et al., 2006).

The different patterns of biomass allocation in forest species can be viewed indirectly by their bigger leaves and tallest individuals compared with congeneric savanna species (Hoffmann and Franco, 2003; Hoffmann et al., 2005; Hoffmann and Franco, 2008). It also can be interpreted as an evolutionary response, since natural selection on forest species may have acted in favor of distinct and opposite resource than savannas (Maracahipes et al., 2018), the light, and thus, in the aerial growth (Dantas et al., 2015; Gignoux et al., 2016; Rodriguez and Maiti, 2016). However, plant height showed a huge nitrogen dependency to achieve the potential growth, especially in forest species, which displayed massive differences when grew in solutions with and without this nutrient, for example. In all treatments with nitrogen absence, the forest species reduced more than 20% of its height compared with savannas species under the same nutritional conditions. The height is recognized as the most important functional trait to compete for light (Gignoux et al., 2016; Moles et al., 2009) and was strongly decreased by nutritional deficiency.

Although the height able the plants to intercept the light, the photosynthetically active organ is the leaf. The capacity of leaves to intercept the light could be changed in many ways, including increasing its area (Duursma et al., 2012). However, the light does not need just to be intercepted, it needs to be used, and in environments with adequate nutritional supply, the protein concentrations, nucleic acids, and energy molecules can increase and enhance the plant’s metabolism (Niinemets, 2010). These molecules have nitrogen and phosphorus in their constitution, and although the plants may still have been under cotyledons

Figure 2. The effect of our four treatments (Com: Complete Hoagland solution; -N: Solution without nitrogen; -P: Solution without phosphorus and -NP: Solution without both macronutrients) in the grow of a congeneric pair of species after 45 days of experiment. The boxes in the left side represent the savanna species (Enterolobium gummiferum), those on the right side, the forest ones (E. contortisiliquum).
contents influence (Milberg and Lamont, 1997; Green and Juniper, 2004), the external nutritional offers were very relevant in diversifying the plants’ leaf areas. Similar to previous traits, nitrogen was also the most important nutrient to increase the leaf area, but the largest leaves in solution with all nutrients, in both biomes, showed a high nutrients dependency to improve efficient light use, including in savannas seedlings.

The efficient light use can be predicted by specific leaf areas, which is usually higher in forest species than in the savannas (Hoffmann and Franco, 2003; Hoffmann et al., 2005; Hoffmann and Franco, 2008). However, we did not find it since the savanna species presented higher specific leaf areas. This difference may have come from our methods, since the greatest physiological problem for plants with large leaf areas is the high-water loss due to transpiration (Taiz et al., 2015), what did not happen in our experiment since we used hydroponics technique. According to Oliveras and Malhi (2016), the savannas and forest congeneric species also present different strategies to deal with water availability gradients. Thus, using hydroponics techniques may have led to our clashing results compared to previous work.

There was one more different strategy adopted by species from these vegetation types. Whereas savanna species displayed the smallest specific leaf area in the treatment with shorter individuals, they also had higher biomass allocation to roots, showing that they were able to keep their morphological characteristics that provide better competition capacity to aboveground resources, even in a life phase in which light incidence is scarce (Hoffmann and Franco, 2008). That trade-off was different on forest species since the same treatment that they presented the shorter individuals also presented the highest specific leaf areas. It seems to be very useful since if the nutritional resources cannot support the growth in height and leave areas, the investment in specific leaf areas on these scenarios can be the best nutritional strategy to enhance their ability to compete for light. When specific leaf area is higher, water loss by transpiration increases (Liu et al., 2017), but it is not a problem in forest environments, where water availability is also higher (Ellison et al., 2017). Thus, when the forest species present less efficient functional attributes in light capture, they still try to display another mechanism that optimizes not only plant growth but also efficiency in light capture (Prado Junior et al., 2015; Liu et al., 2017), highlighting their adaptive strategies to survive in closed environments.

The functional traits difference displayed between savanna and forest species was strongly influenced by the nutritional conditions suggesting different strategies on typical species from those biomes. It highlights the different pressures experienced by these different functional groups throughout their evolutionary history (Hoffmann and Franco, 2008; Silva et al., 2013). Our results pointed out that nitrogen was the most important nutrient, shaping the functional traits in seedlings from both savanna and forest species. As nutrient availability — especially nitrogen — is changing, one biome may be favored over the other, altering the structure and functioning of the savanna and forest mosaic (Hill and Southworth, 2016). Forest encroachment in savannas landscapes is a global phenomenon already described by changes in the fire regime (Staver et al., 2011; Stevens et al., 2017). It may also be related to changes in nitrogen and phosphorus availability.

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