When to make partners in the city: phosphorous enrichment disrupt the partnership between the invasive herb Ruellia nudiflora (Acanthaceae) and arbuscular mycorrhizal fungi in a tropical urban environment.

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Abstract

The mutualistic relationship between plants and arbuscular mycorrhizal fungi (AMF) is essential for optimal plant nutrition, enabling the plant to better withstand biotic and abiotic stressors and enhancing its chances of survival, reproduction, and colonization of new environments. Urban soil nutrient enrichment may reduce the benefits of AMF for plant nutrition, potentially reducing interaction with AMF in urban environments. Here, we test this prediction by studying how urbanization alter the plant-AMF interaction between the invasive herb Ruellia nudiflora (Acanthaceae) and AMF in Mérida city. We collected soil and plants from deep urban sites (DUS; e.g. sidewalks), open urban sites (OUS; parks), and rural sites (RS) to analyze the soil nutrient content, plant morphology, AMF-colonization rates, spore density, richness, and diversity. Unexpectedly, DUS showed the lowest soil nutrient concentrations except for phosphorus. Higher phosphorus levels in these sites reduced AMF colonization, supporting the prediction of reduced plant-AMF interactions in urban environments. We discovered that potassium affects the plant-AMF association, an understudied effect. Finally, urban plants produced smaller and more compact roots than rural plants, and no differences on AMF communities were found between urban and rural environments. To gain a better understanding of how AMF contributes to plant colonization in urban environments, further studies are required.

Introduction

Urban areas represent ca. 0.5% of the land area in the world, in which around 55% of the human population currently lives (Seto *et al.* , 2012; Liu *et al.,* 2020). However it is projected that by 2030, the urban coverage will increase by 50%, mainly due to the fast urbanization occurring in developing countries (Seto *et al.* , 2012). This rapid expansion of the urban ecosystem around the world can affect the ecology and evolution of urban dwellers by imposing new and extreme environmental conditions, such as impervious surfaces, high temperature, high pollution (soil, air, water, light, and sound), and high nutrient availability (Kaye *et al.* , 2006; Grimm *et al.* , 2008; Menberg *et al.* , 2013). Changes in abiotic urban attributes can cause alterations in the biotic component of the urban environment, for instance, by affecting the abundance and diversity of species within urban communities (Grimm *et al.,* 2008), or by reducing the intensity or affecting the nature of ecological interactions (e.g. shift from mutualism to antagonisms or vice versa) (Miles *et al.* 2019; Irwin *et al.,* 2020). In this way, disentangling the roles of urban abiotic factors on the ecology of biotic interactions is key to understanding the causal relationship that drives adaptation to urban environments. For instance, soil nutrient enrichment in urban environments can alter the soil microbiota that plays a central role in plant physiology, above-ground interactions, and plant fitness (Mejia-Alva *et al.* , 2018; Irwin *et al.* , 2020).

Urban abiotic conditions can also alter the microbiota communities (Weerasundara *et al.* , 2017), its relationship with plants (Lin*et al.* , 2021), and potentially condition the function of the urban ecosystem (Harris,

1991). Urban soils are often highly enriched due to atmospheric deposition, combustion, importation of food, and fertilizer application (Harris, 1991; Kaye *et al.* , 2006). These factors condition the interaction among nutrients, for instance, high calcium (Ca) concentrations can increase soil pH, which in turn can enhance potassium (K), phosphorous (P), and nitrogen (N) availability (Osman, 2013). Specifically, direct and indirect effects between soil components that ultimately alter P and N availability can cause important alterations in the mutualistic plant-arbuscular mycorrhizal fungi (AMF) interaction by altering the cost-benefit balance that drives this interaction (Salvioli di Fossalunga & Novero, 2019; Irwin *et al.,* 2020).

The mutualistic arbuscular mycorrhiza interaction is 460 *myr* old, and 80% of the plant species can form an association with arbuscular mycorrhizal fungi (AMF) (Smith & Smith, 2011). AMF are obligated symbionts, which provide increased availability of N and P to plants in exchange for carbon (Smith & Smith, 2011), as well as conferring the plant with resistance to various types of biotic and abiotic stresses, and increasing plant survival and reproduction (Ramos-Zapata *et al.,* 2010; Mejia-Alva *et al.* , 2018; Wu & Zou, 2017). Nevertheless, this association, which can be measured as the AMF-colonization rate, can be disrupted by increasing P and N soil concentrations (Salvioli di Fossalunga & Novero, 2019), which reduces the benefits that plants obtain from the association with AMF (Smith & Smith, 2011). Such reduction in plant-AMF association can be expected in urban environments if P and N are enriched (Kaye *et al.,* 2006). Interestingly, while previous studies have found that plants in urban soils interact less with AMF (Tyburska *et al.* , 2013), and that urban soils show lower spore richens, diversity, and density, than rural soils (Cousins *et al.,* 2003), there are few studies that explore the urban soil properties that affect the plant-AMF association (Egerton-Warburton & Allen, 2000; Wiseman & Wells, 2005; Buil *et al.* , 2021).

Ruellia nudiflora (Acanthaceae) is a perennial herb that interacts with AMF and can grow in urban and rural environments (Tripp, 2007; Ramos-Zapata *et al.,* 2010). Previous research has detected genetic variation in *R. nudiflora* for this association as well as AMF positive effects on fitness (Ramos-Zapata *et al.* , 2010; Mejia-Alva *et al.,* 2018). For instance, AMF increased by 18% plant size and 15% fruit production of *R. nudiflora* (Mejia-Alva *et al.,* 2018), and indirectly increased fitness by reducing seed predation from the moth *Tripudia paraplesia* (Noctuidae) by 50% (Mejía-Alva *et al.* , 2018). All this evidence suggests that AMF can play a key role in the incursion of *R. nudiflora* into urban environments and become a useful model to understand the ecological and environmental effects of the urban environment on plant-AMF interactions.

The main goal of this research is to investigate the effects of urbanization on the mutualistic relationship between *R. nudiflora* plants and AMF, and to test the prediction that the mutualistic interaction plant-AMF should be reduced in enriched urban soil (Irwin *et al.,* 2020). For this, we focus on answering three questions. 1) Do urban and rural soils differ in their abiotic properties and in *R. nudiflora* plant traits (e.g., biomass, root shape)? 2) Do *R. nudiflora* 's AMF-colonization rates, spore density, and diversity vary between rural and urban environments? And, lastly, 3) How are AMF-colonization rates on *R. nudiflora* associated with soil properties, and to what extent are these associations affected by urbanization?

Materials and Method

Study species

Ruellia nudiflora (Engelm. & A.Gray) Urb. (Acanthaceae) is a perennial herb native to Texas (Turner, 1991), commonly found in urban and rural areas (Fig. 1), with a wide distribution from southern United States to southern Mexico and Central America (Tripp, 2007). It is a self-compatible species that produces flowers with open corolla (chasmogamous), allowing outcrossing by insect pollination, and flowers with closed corolla (cleistogamous), preventing outcrossing (Tripp, 2007). Common herbivores include the leaf-eating caterpillars *Anartia jatrophae* and *Siproeta stelenes* (Nymphalidae) (Ortegón-Campos *et al.* 2009), and the seed predator *Tripudia paraplesia* (Noctuidae; Abdala-Roberts *et al.,*2016), which in turn is attacked by several parasitoid species from three wasp families (Braconidae: four species, Ichneumonidae: one specie, Pteromalidae: two species) and one fly species (Tachinidae) (Abdala-Roberts *et al.* , 2016). Ant-aphid interactions have also been observed on *R. nudiflora* in both rural and urban areas (pers. obs.). Studies on the *R. nudiflora* -AMF interaction have found that the average colonization rate by AMF is 52%, and this

association has a positive effect on growth rate and plant fitness (Ramos-Zapata *et al.* , 2010; Mejia-Alva *et al.* , 2018), as well as an increase in plant cover and a reduction of attacked fruits by seed predators (Mejia-Alva *et al.* , 2018). Previous research has studied the effect of soil conditions on *R. nudiflora* fitness and found a significant effect on survival (Ortegon-Campos *et al.* , 2012). All this information shows that *R. nudiflora* is a plant facing a complex multispecific environment (herbivores, third-trophic level, AMF interactions) which can drive adaptive evolution; however, there is a lack of studies evaluating the effect of urban conditions on*R. nudiflora* and its ecological interactions.

Study area

Merida city is the capital of the state of Yucatan, Mexico (city code given by UN 2018: 21851), which was conquered and funded in 1542 (Maya original name T'ho) by the Spaniards. Merida is located at 20.9667 degN, 89.6167 degW, and at 10 m.a.s.l. with warm sub-humid weather with a rainy season spanning from June to October (Merida City Council, 2018). The mean annual rainfall is 959 mm, and the mean annual temperature is 26–27.5 degC, with the mean maximum temperature occurring in April–May (37.5–41 degC), and the minimum (16 *C) in January–February (Merida City Council, 2018). Merida is the largest and most populated tropical city in the Yucatan Peninsula, which, since 1970, has experienced a sustained urban expansion due to flat topography, groundwater availability, and weak enforcement of urban growth regulation. The urban expansion in the 2001-2018 period has promoted the deforestation of 5,413.2 ha (in average of 205 ha/year), driving the emergence of a heat island effect, increasing temperatures in 2.36–3.94 degC after deforestation (Carrillo-Niquete *et al.,* 2020). In 2020, the population of the city reached 921,771 people, representing 92.6% of the total population of the municipality, and 39.7% of the state's total population (INEGI, 2020). Finally, the growth rate of Merida city in 2020 was 1.75 (UN), and currently 86% of the population of Yucatan lives in urban areas (INEGI, 2020). Adjacent rural areas are characterized by edges of agricultural fields and roadside ditches.

Field sampling.

To test for differences in soil properties, plant attributes, and the*R. nudiflora* -AMF interaction (i.e. colonization rate) between urban and rural environments (questions 1 and 2), and to explore the relationship between plant and soil attributes on colonization rates (question 3), we collected *R. nudiflora* individuals, including their roots and their rhizospheric soil in urban and rural environments (Fig. 1, Fig. S1). Specifically, for the urban environment, we sampled in the oldest parts of Merida city, including sampling sites at least 3 km inside the ring highway (Fig. S1). We reduced the high environmental heterogeneity of the city creating two extreme categories where *R. nudiflora* can be found: deep urban sites (hereafter DUS), and open urban sites (hereafter OUS). DUS are sites with a high degree of impervious surface and low plant density in which *R. nudiflora*can be found growing in cracks of sidewalks and road asphalt (Raupp*et al.* , 2010);meanwhile OUS include sampling sites in parks and road medians strips where *R. nudiflora* can also be found (Fig. 1). Rural sites (hereafter, RS; Fig. 1) were sampled in rural areas as far away from human settlements as possible, and sampling sites were situated at least 3 km from the city ring highway (Anillo Periferico; see Fig S1). In urban (DUS and OUS) and rural environments (RS) the spatial distribution of the sampling sites were designed to be equally distributed across each cardinal orientation; however due to lack of plants in certain areas or difficult access (e.g. lack of roads in the rural environment) the sampling design was not fully balanced. We ensure that the distance between the nearest sites was at least 300 meters (Fig. S1). For each environment (DUS, OUS and RS) five plants were sampled in 20 sites (3 environments/20 sites/5 plants) for a total of 300 sampled plants.

Sample processing and data collection

Plant morphometrics: In those plants collected in the field we recorded the number of branches, plant height, number of primary roots, length of the longest root, and root volume. Sampled plants were dried at 60oC for 72 hrs., to record the dry biomass of roots and shoots.

AMF-colonization rates and spore identification: Thin secondary roots were collected to determine the root colonization rates from AMF, using a modified version of the Trypan Blue technique (see Ramos-Zapata*et*

al. , 2011). In every site, we pooled the rhizospheric soil from each of the five sampled plants per site, and then a sample of 100 g of this composite sample was used to extract spores using the wet sieve method (see Mejia-Alva *et al.* , 2018). Afterward, the spores were placed in microscope slides with PVLG-Melzer solution to estimate spore density, richness, and diversity of AMF. Viable spores were counted and identified as morphospecies based on the color, presence, and type of ornamentation, and presence of bulbs using INVAM species descriptions as a guide (INVAM, 2017).

Soil nutrients: From the pooled soil collected per site, a sub-sample of 50 g was taken and sieved through a 1mm mesh to perform soil nutrient and pH analyses. Nitrogen (N) and inorganic phosphorus (P) were assessed by the Kjeldahl method and Olsen method (see Estrada-Medina *et al* ., 2016), respectively. Meanwhile potassium (K), sodium (Na), and calcium (Ca) were determined by flame photometry (Helmke and Sparks, 1987). pH values were obtained with potentiometry (see Estrada-Medina *et al* ., 2016).

Statistical analyses

Contrast between urban and rural soil, plant traits and AMF variables: To test for differences between urban and rural environments in soil and plant traits (question 1), and AMF variables (AMF-colonization rates, spore density, and diversity; question 2), we ran independent one-way ANOVAs to test for the effect of the environment factor (three levels: RS, DUS, OUS). Variables such as soil properties (e.g. N concentration, pH), spore density, richness, and diversity were pooled at the site level; spore diversity was calculated using the Shannon-Wiener diversity index. On the other hand, because replication for variables such as plant morphometrics and AMF-colonization rates were at the plant level, we implemented the *lmer* function from package *lmer4* in R (Bates *et al.* , 2015) to perform linear mixed models considering *environment* and *site* as fixed and random factors, respectively. The significance of the fixed effect was evaluated using a Type II Wald's test running the *Anova* function from the *car* package in R (Fox & Weisberg, 2019), assessing the significance of the random effect with a likelihood-ratio test (LRT). To discard potential spatial autocorrelation, Mantel's tests were used to assess the correlation between geographic distance and Euclidean distance for each variable recorded using the package *ade4* in R (Chessel *et al.* , 2004); however, in none of the considered dependent variables spatial autocorrelation was detected. To test for changes in community composition based on spore morphospecies between environments, we conducted a permutational analysis of variance (PERMANOVA) with 999 permutations using the *adonis* function from the *vegan* package in R (Oksanen *et al.* , 2020). Finally, a Principal Component Analysis (PCA; *prcomp* , R), based on a correlation matrix, was used to test for differences in the multidimensional variation of soil properties and plant attributes between each environment (question 1). A one-way ANOVA tested the *environment* (RS, DUS, OUS) effect on PC1. This PC1 summarized the variation on soil nutrient concentration (N, P, K, Na, and Ca) and pH. Before PCA, we equalized P and Ca variances by dividing raw values by 100.

Soil and AMF associations: To explore the soil properties that may predict AMF-colonization rates on roots of *R. nudiflora*(question 3), we performed pairwise correlations between AMF-colonization rate and soil attributes at the global level (i.e. using data from all environments) as well as for each environment separately. As a next step, we used the Z-scores of PC1 (see previous section) to explore the association between PC1 and AMF-colonization rate (using pooled data). However, because PCA visual inspection and previous statistical analyses on the first principal component (PC1; see previous section) indicated strong differences in Z-scores between urban and rural environments we ran an independent linear model (i.e. AMF-colonization rate ~ PC1) within each environment.

Structural equation modeling: Because analyses based on principal components obscure the relationship between soil attributes mediating the levels of AMF-colonization rate, we implemented Structural Equation Modelling (SEM) using *lavaan* package in R (Rossel, 2012), and tested for contrasts between urban (DUS, OUS) and rural (RS) environments using a multigroup test. We used SEM approach to disentangle the direct and indirect effects that soil nutrients and pH have on AMF-colonization rate (question 3). We designed an initial causal model (Fig. S2) based on previous knowledge of soil properties and nutrient interactions (Fig. S2). We considered Ca as one of the main drivers of pH, which in turn can affect K, P, and N availability (Osman, 2013). However, the two later macronutrients (P and N) have been reported to negatively affect

K availability (Osman, 2013; but see Dibb & Thompson, 1985). In turn, it is expected that N and P will have important and negative effects on the AMF-colonization rate (Salvioli di Fossalunga & Novero, 2019; Chen *et al.* , 2020). Finally, Na can also increase the benefit of the association with AMF in the context of salinity stress (Evelin *et al.* , 2009), and, in soils with low K availability, AMF can play an important role to improve plant K nutrition (Garcia & Zimmermann, 2014). The goodness-of-fit of the initial causal and alternative models (Fig. S2) was assessed with an LRT to test the null hypothesis that the predicted covariance matrix of the model is not different from the observed (Iriondo *et al.* , 2003). A significant χ^2 indicates that the model does not fit the observed covariance matrix. Because a good-fit model may result from an inadequate statistical power (Mitchell, 1992), we calculated additional indices of goodness-of-fit, such as the Comparative Fit Index (CFI; cut-off good fit [?] 0.95), the Root Square Mean Error of Approximation (RMSEA; cut-off good fit [?] 0.06), and the Standardized Root Mean Square Residual (SRMR; cut-off good fit [?] 0.08), which are insensitive to sample size (Hooper *et al.,* 2008). All these indices were estimated using the *fitMeasures* function in the *lavaan* package in R (Rossel, 2012), and were used in the process of model selection.

Once we obtained a fitted and general causal model, we ran a multigroup test to assess whether path coefficients contrast between DUS, OUS, and RS environments. The multigroup test imposes cross-group constraints on the path model regression coefficients, and then compared with an LRT, the constrained and unconstrained models using the *lavTestLRT*function (*lavaan* package in R; Rossel, 2012). In particular, we used the function *lavTestScore,* a Lagrange Multiplier Test (LMT), as a guide to identify a set of constrained path coefficients that, if released, would result in a significantly better model (i.e. a lower goodness-of-fit $\chi 2$; Bentler, 1989).

All statistical analyses were performed using R version 4.0.3 (R Core Team, 2020). Furthermore, all analyses, were evaluated with transformed variables when needed to meet the normality assumption.

Results

1. Do urban and rural soils differ in their abiotic properties and in R. nudiflora plant traits (e.g., biomass, root shape)?

All soil characteristics were significantly different between the most extreme environments RS and DUS (Table 1). Particularly, we found that DUS showed lower N concentrations when contrasting against the other environments (64% and 49% lower than RS and OUS, respectively) (Table 1; Fig. 2a). P was the only macronutrient found enriched in DUS, showing 1.7 and 2.2 times higher concentrations than in RS and OUS, respectively (Table 1; Fig. 2b). For RS and OUS, both N and P concentrations were similar between environments (Fig. 2a, b). K concentrations were the highest in OUS (12.3 and 2.3 times higher than DUS and RS) while DUS presented the lowest K concentrations (Fig. 2c). Similarly, OUS also showed the highest concentration of Ca and Na. OUS and RS showed a similar pH, but higher than the recorded in DUS (Table 1).

When exploring differences in plant attributes among environments, we found that plants growing in OUS showed the lowest total biomass, but we did not find differences between RS and DUS (Table 1). Above-ground (shoot) biomass in RS was not different from OUS and DUS; however, plants growing in DUS showed 1.34 times higher shoot biomass than plants growing in OUS (post-hoc Tukey HSD test; Table 1). A similar pattern was found in plant height, and number of branches (Table 1). When below-ground (root) traits were evaluated, we found that in general, plants growing in urban environments (i.e. DUS and OUS) were significantly smaller in all root attributes than plants growing in RS (Table 1). Particularly, when contrasting the two more extreme environments, DUS vs RS, we found that DUS plants had 82% less root biomass, 45% less root volume, 72% shorter roots, and 23% less primary roots than RS plants (Table 1). Meanwhile, no differences were detected in root dry biomass between plants collected in OUS and DUS. Nevertheless, we detected that DUS plants have higher root volume (60%) but less and shorter primary roots than OUS plants (Table 1).

Contrasting patterns of covariation within soil and root attributes were detected among urban (DUS and

OUS) and rural (RS) environments. The first principal component from the PCA including soil attributes ($PC1_{soil}$) accounted for 62.9% of the variation and showed that pH, N, Ca, K, and Na covary positively among them and negatively with P concentration (Table S1; Fig. 3a). A visual inspection of ellipses in the biplot (Fig. 3a) illustrate contrasting variation between DUS, OUS, and RS environments, placing DUS soils in the higher extreme of the $PC1_{soil}$ where higher P concentration are present while other macronutrients are scarce ($DUS_{PC1\ soil}$ = 2.39 ± 0.12^a; $OUS_{PC1\ soil}$= -2.00 ± 0.15^c; $RS_{PC1\ soil}$ = -0.38 ± 0.18^b; $F_{environment2,\ 57}$ = 218.71, $P < 0.001$).

The first principal component of the PCA including root attributes (root length, number of primary roots, root volume) was interpreted as a new variable describing root size ($PC1_{root\ size}$; Table S2), which accounted for 64.3% of the variation. Based on this new variable, we detected that rural plants have the biggest roots and urban plants the smallest ones; in particular, plants from OUS produced the smaller roots ($RS_{PC1\ root\ size}$ = 0.91 ± 0.16^a; $DUS_{PC1\ root\ size}$ = -0.19 ± 0.11^b; $OUS_{PC1\ root\ size}$ = -0.69 ± 0.08^c;$F_{2,\ 295}$ = 65.27, $P < 0.001$; Figure 2b). On the other hand, we also found a contrast between environments in root shape represented by $PC2_{root\ shape}$(accounting for 25.1% of the variation; Table S2). In particular, we found that plants growing in DUS have a higher number of short and thicker primary roots than roots in OUS and RS ($DUS_{PC2\ root\ shape}$= -0.49 ± 0.68^b; $OUS_{PC2\ root\ shape}$= 0.29 ± 0.06^a; $RS_{PC2\ root\ shape}$= 0.23 ± 0.10^a; $F_{2,\ 295}$ = 30.38, $P < 0.001$). This indicates a contrasting root architecture, where DUS and OUS roots are smaller than RS roots, but DUS roots are shallower than the ones in RS and OUS (Fig. 3b).

2. *Do R. nudiflora's AMF-colonization rates, spore density, and diversity vary between rural and urban environments?*

Root AMF-colonization rates in RS plats were 2.6 and 1.9 times higher than in DUS and OUS plants, respectively (Table 1; Figure 1d); however, DUS and OUS AMF-colonization did not differ significantly. Although spore density, richness, and diversity were higher in RS, it was not significantly different from DUS and OUS (Table 1; Fig. 2e, f). Furthermore, a PERMANOVA did not reveal differences in community composition between environments (R^2 = 0.0029,$F_{2,55}$ = 0.08, P = 0.941). Neither spore richness, diversity, and density were associated with any soil chemical attribute (results not shown), except for a negative effect of P on spore diversity (β = -0.004, R^2 = 0.093,$F_{1,58}$ = 5.95, P = 0.0178).

3. How are AMF-colonization rates on R. nudiflora associated with soil properties, and to what extent are these associations affected by urbanization?

Based on global pairwise correlations (n_{pooled} = 60), we detected that the AMF-colonization rate was only positively associated with N concentration (r = 0.37, $P < 0.001$) (Table S3). Nevertheless, the patterns of associations between soil properties and AMF-colonization rate changed when pairwise correlations were estimated by environment (Table S4, S5 & S6). In the DUS environment, the significant association between N and AMF-colonization was lost and a positive effect of K and Ca was detected (r = 0.46, df = 18, $P <$ 0.05; r = 0.90,df = 18, $P < 0.001$). Meanwhile, in the RS environment only a negative association between P with AMF-colonization was detected (-0.54, $P < 0.05$; Table S5). In contrast to the other environments, OUS did not show any association between AMF-colonization and soil characteristics (Table S6). Table S3 to S6 also show changes in patterns of associations between soil properties which are summarized in $PC1_{soil}$ to explore what variables may be predicting AMF-colonization rate in *R. nudiflora*under the effect of urbanization.

We discovered that an increase in $PC1_{soil}$ reduced AMF-colonization rates in plants growing in DUS, the environment with higher P concentration(Fig. 4). This negative association between the Z-scores from $PC1_{soil}$ suggest that the simultaneous increment in P concentrations and the reduction on N, K, Ca, Na and pH, decreases the AMF-colonization rates on *R. nudiflora* (β = -0.232 ± 0.073, P = 0.017,R^2_{adj} = 0.238; Fig. 4). No other association between $PC1_{soil}$ and AMF-colonization rate was detected neither in OUS and RS environments (RS, β = -0.026 ± 0.044, P = 0.555,R^2_{adj} = -0.035; OUS, β = 0.069 ± 0.051, P = 0.188,R^2_{adj} = -0.044; Fig. 4). No statistical evaluation on the effect of the environment on the association between $PC1_{soil}$ and AMF-colonization rates ($PC1_{soil}$ × environment) was possible due to strong collinearity

between $PC1_{soil}$ Z-scores and the categorical factor e*nvironment* . Even though this approach suggests the importance of P on AMF-colonization rates, such effect can be confused with the reduction on other soil attributes (N, K, Ca, Na, and pH) along the $PC1_{soil}$ axis. To disentangle the specific contribution of each soil attribute we implemented SEM.

The SEM that supported the results below is a reasonable description of the processes that generate the observed correlations among variables (χ^2_2 = 2.661, P =0.264, CFI = 0.99; RMSEA = 0.074; SRMR = 0.053; Fig. 5). Overall, our findings showed that P concentration reduced AMF-colonization rates in RS and that this effect was unaffected by variation in other soil characteristics. (Fig. 5). Moreover, the negative effect of P on AMF-colonization rate was different among environments ($LRT_{multigroup\ test}$ = 3.985, df = 1,P = 0.046; Fig. 5). Furthermore, regardless of soil origin, we found that N concentration had a positive effect on AMF-colonization rate ($LRT_{multigroup\ test}$ = 0.26, df = 1,P = 0.011; Fig. 5). In the case of K, we detected that while it increased AMF-colonization rate in DUS, it reduced colonization in RS and OUS environments ($LRT_{multigroup\ test}$ = 6.013,df = 1, $P < 0.05$; Fig. 5). Such shifts in association between K and AMF-colonization altered the N indirect effects that run through K due to an important association between N and K (path coefficient: $\rho_{\text{AMF-colonization rate.K}}$ = - 0.17 ± 0.06). For instance, in RS, N had a negative indirect effect that reduced the total positive contribution of this macronutrient on AMF-colonization rate (indirect effect $\rho_{\text{K.N}} \times \rho_{\text{AMF-colonization rate.N}}$ = 0.17 × -0.45 = - 0.078; N total $\text{effect}_{\text{direct + indirect effect}}$ = 0.18); a similar pattern was observed in OUS (indirect effect $\rho_{\text{K.N}} \times \rho_{\text{AMF-colonization rate.N}}$ = -0.043; N total effect = 0.22). Contrary to such negative indirect effects, we found in DUS a N positive indirect effect, increasing the total effect of N on the plant-AMF interaction (indirect effect $\rho_{\text{K.N}} \times \rho_{\text{AMF-colonization rate.N}}$ = 0.225; N total effect = 0.515). Finally, we also detected that pH affects K concentration negatively in RS and DUS, but positively in OUS environments ($\text{LRT}_{\text{multigroup test}}$ = 5.774, df = 1, P = 0.016), and that these changes can impact the indirect effect that pH have via K in AMF-colonization rates (Fig. 5).

Discussion

Our results support that urbanization alters soil nutrient-enrichment, but in the opposite direction to what is expected (Irwin *et al.,*2020). In the most extreme urban environment (deep urban sites; DUS) we found the lower enrichment nutrition levels, except for P. This high P concentration was responsible for reducing the interaction between*R. nudiflora* and AMF in DUS. Overall, our results confirm that urbanization can alter soil nutrient-enrichment (Kaye *et al.* , 2006), that P, K and N concentrations drive plant-AMF interaction (Garcia & Zimmermann, 2014), that AMF-colonization rates are reduced in urban environments, and that changes in soil nutrient-enrichment due to urbanization affect the plant-AMF interaction (Newbound *et al.,*2010; Irwin *et al.,* 2020). Even though we detected such alterations in the AMF-colonization rates, no differences were detect for spore density, richness, diversity, and community composition. Finally, we found that plants growing in the most extreme urban environment underneath of impervious surface, such as downtown sidewalks (which are characteristics of the deep urban sites; DUS), have short and ramified roots (Fig. 3b).

1. Do urban and rural soils differ in their abiotic properties and in R. nudiflora plant traits (e.g., biomass, root shape)?

The literature indicates that urban soils are importantly affected by anthropogenic activity (Wiseman & Wells, 2005; Harris, 1991; Kaye*et al.* , 2006; Irwin *et al.,* 2020). Nutrient enrichment, such as P and N (Kaye *et al.* , 2006), high temperatures (Menberg*et al.* , 2013), compacted soil, and the presence of impervious surfaces (Day *et al.* , 2010) can lead to a reduction in plant size in urban environments (Yakub & Tiffin, 2017), as well as to a reduction of soil mutualistic interactions (Newbound *et al.,* 2010; Irwin*et al.* , 2020). Our results show that DUS tends to have less nutrient concentrations than RS and OUS, except for P. These findings do not match with the general expectation that urban soils should be nutrient enriched (Wiseman & Wells, 2005; Kaye *et al.,* 2006; Irwin *et al.,* 2020). Our results highlight the high microenvironmental heterogeneity within cities that are under different anthropogenic management, such as parks and road median strips which have higher organic matter that could explain the enriched soil in OUS (Irwin *et al.* , 2020).

The difference between the two urban environments (DUS and OUS) can be attributed to DUS impermeable surfaces (Day *et al.* , 2010) that reduce P runoff and lixiviation, which is expected to be higher in OUS as well as in RS (Estrada-Medina, 2016). Meanwhile, the increase in P concentration in DUS may be caused by combustion and atmospheric deposition (Kaye et al., 2006). A previous study along a urban-rural transect in New York city, also found higher soil nutrient enrichment in rural than in urban soils (Zhu & Carreiro, 2004), concluding that the low N enrichment in cities was due to reduced nitrification from bacteria due to greater concentrations of heavy metals. In the same study, they explained the reduction in P in cities might be due to reduced organic mass inputs; however, this does not explain the higher P concentrations we found in DUS in this study. Soils in the region of Mérida city are poor in nutrients due to high soil permeability and lixiviation (Estrada-Medina, 2016); however, because Fabaceae species are dominant in the Yucatan forests, the presence of N-fixing bacteria on legumes root nodules could explain higher N concentration in rural areas (Rivero-Villar *et al.* , 2018). Nevertheless, these arguments cannot explain the park and road medians nutrient enrichment since Fabaceae species are missing or in a lower abundance than in rural areas (pers. obs.). High organic matter and bacteria in the rural soils (Estrada-Medina, 2016) could also explain nitrogen enrichment for both RS and OUS, although when considering the higher heavy metal concentrations in OUS we would not expect an increase in bacterial nitrification (Zhu & Carreiro, 2004).

Environmental contrasts between urban and rural areas are predicted to shape the phenotypic expression of functional traits (Cheptou & Lambrect, 2020). In plants, contrasting differences in several plant morphological attributes, have been reported (see Yakub & Tiffin, 2017), but no root morphology attributes have been explored. It is expected that urban conditions, such as higher temperature, soil compaction, and impervious surfaces, would promote reduced root growth due to reduced root elongation and fine root production (Day *et al.* , 2010). In this study, we confirmed this prediction by finding smaller and more compact roots in both urban environments, which can be explained by more compact and shallower soils (pers. obs.). However, given the connection between root functionality and nutrient and water intake, it would have been useful to evaluate secondary root properties such as diameter, length, density, and biomass. (Karliński *et al.* , 2014; Freschet *et al.,* 2017), which have been found to be different between deep urban and rural trees (Karliński *et al.* , 2014). A previous study in *R. nudiflora* found that this long-lived herb reallocates carbon reserves into roots, as a compensatory mechanism, and that higher investment in root biomass favored reproductive compensation to herbivory (Rivera-Solís *et al.,* 2012). Interestingly, for *R. nudiflora* , individuals living in the city, such compensatory mechanisms might not be relevant against folivory since the incidence of foliar damage is lower than in rural areas (pers. obs.), but it can play an even more important role to invade the city as a strategy to deal with strong and regular mowing due to street maintenance (around every month in the rainy season). This and other ecological contrasts (e.g. soil compaction) set the basis to explore for root phenotypic divergence and their implications on above-ground interactions (Bardgett *et al.* , 1998); however, there is currently no experimental research that has explored root phenotypic divergence driven by urbanization and their physiological and ecological implications. In this context, the use of common gardens or reciprocal transplant experiments will be key to explore such divergence and the importance of phenotypic plasticity (Nuismer & Gandon, 2008).

2. *Do R. nudiflora's AMF-colonization rates, spore density, and diversity vary between rural and urban environments?*

In general, urban soil conditions have been shown to negatively affect fungal communities including ectomycorrhizal fungi (ECM) and AMF; however, there are still few studies focusing exclusively on AMF (Newbound *et al.,* 2010). Most studies support our finding that plant populations growing in urban areas have lower rates of AMF-colonization than rural populations (see Egerton-Warburton & Allen, 2000; Wiseman & Wells, 2005; Tyburska *et al.* , 2013), while only one study found similar colonization rates between urban and rural environments in AMF (Karliński *et al.* , 2014). Interestingly, all these previous studies were conducted in tree species, and despite some of these studies recording multiple soil attributes (e.g. Tyburska*et al.,* 2013), only few have explored which soil chemical component drives the intensity of the interaction (Egerton-Warburton & Allen, 2000; Wiseman & Wells, 2005; Buil *et al.* , 2021). In this way, our study is the first to focus on an invasive perennial herb, and to explicitly disentangle the implication of urbanization on the functional role

of macronutrients affecting this mutualistic interaction (see next section).

In relation to mycorrhizal communities, previous studies have assessed changes due to urbanization in ECM communities (Ochimaru & Fukuda, 2007) and in AMF communities (Egerton-Warburton & Allen, 2000; Cousins*et al.,* 2003; Buil *et al.,* 2021). In general, the trend is a reduction in AMF richness, diversity, and density of propagules in urban environments (Egerton-Warburton & Allen, 2000; Cousins *et al.,* 2003; Ochimaru & Fukuda, 2007; Lin *et al.,* 2021; Buil*et al.,* 2021). In contrast, we did not find differences in diversity or community composition between the urban and rural environments. Similarly, to the case of AMF-colonization rate, there are few studies that have evaluated the relevance of soil or other properties to explain changes in urban AMF communities (Egerton-Warburton & Allen, 2000; Buil *et al.,* 2021). While Buil*et al.,* (2021) recorded multiple soil chemical attributes they did not statistically tested for any associations with AMF community parameters (e.g. diversity); however, they did find lower AMF richness and diversity in the more extreme urban soils, and that both were affected by vegetation coverage. On the other hand, Egerton-Warburton & Allen (2000) found, in an anthropogenic N deposition gradient, that enrichment in N reduced AMF spore richness and diversity. In our study, despite the detected contrast in soil chemistry between urban and rural soils, we did not find differences in either richness or diversity of AMF spores, and neither in community composition, but we did find a negative association between P concentrations and spore diversity. The lack of differences in spore density between urban and rural environments could be due to spore dispersion by vectors, such as wind, insects, birds, and pedestrians (Buil *et al.* , 2021). Contrastingly, Egerton-Warburton & Allen (2000) found that N enrichment due to urbanization reduced spore density. Further experiments should be designed to explore differences in AMF soil inoculum potential (Wiseman & Wells, 2005; Ramos-Zapata *et al.,* 2011) between urban and rural soils to rule out the potential of low soil inoculum as a possible explanation for the decreased AMF-colonization rate shown in both urban environments (Buil *et al.* , 2021). The use of metagenomic approaches will be key to gain knowledge on how soil microbiome communities are affected by urbanization and to understand the role that particular taxonomic AMF groups might play in plant colonization and adaptation to urban environments (Unterseher *et al.* , 2011).

3. How are AMF-colonization rates on R. nudiflora associated with soil properties, and to what extent are these associations affected by urbanization?

In our study, the recording of several soil chemical attributes and the combined use of different statistical approximations allowed us to detect association patterns between soil chemicals and the plant-AMF interaction. First, a PCA detected a positive pattern of association between a set of soil chemical properties (pH, N, K, Na, Ca) that were negatively associated with P concentrations ($PC1_{soil}$). In DUS, where P concentrations were the highest, we detected a reduction in AMF-colonization as P concentration increased and N, K, Ca, Na, and pH were reduced ($PC1_{soil}$). This result suggests that P plays a key role in reducing plant-AMF interaction as previously reported (Smith & Smith, 2011; Chen *et al.,* 2020), or that the reduction on the other soil properties were mediating the interaction. Also, this result indicates that it would be crucial to distinguish the indirect and direct effects of each variable influencing the *R. nudiflora* -AMF interaction, using a SEM approach and considering the patterns of covariation of the chemical properties.

With a SEM approach we were able to detect and confirm that P concentration played a key role in reducing the AMF-colonization rates in *R. nudiflora* , as it was expected (Salvioli di Fossalunga & Novero, 2019; Chen *et al.,* 2020; but see Treseder & Allen, 2002). Our results indicate that a negative effect of P on AMF-colonization rates was observed in DUS and RS, but while in DUS the higher P concentrations result in a reduction of the AMF-colonization rates, the lower concentrations of P promotes higher AMF-colonization rates in RS. While this explains the results found in DUS and RS, for OUS we found low P concentrations and low AMF-colonization rates, reflected on the positive effect of P concentrations on AMF-colonization in the SEM. Chen *et al.* (2020) found a reduction in the magnitude of the effect of P on AMF-colonization rates when native plants were competing with invasive plant species, which have a greater negative effect. It is possible that, in OUS, *R. nudiflora* encountered greater competition, which changed the effect that P had on the plant-AMF interaction. Furthermore, contrary to previous evidence (Egerton-Warburton & Allen,

2000; Karliński *et al.,* 2014), we found a consistent positive effect of N concentrations on AMF-colonization rates, which can be expected in N-limited soils. Moreover, similar positive effects of P on AMF can also occur in P-limited soils (Treseder & Allen, 2002). Such positive effect of P and N on AMF-colonization can suggest that AMF are not only constrained by carbon supplied by plants but also by soil nutrient availability (e.g. N and P), and that below a certain threshold the addition of P and N can have positive effects on AMF (Treseder & Allen, 2002).

While the above studies can explain the positive effect in N among the sampled environments (i.e. DUS, OUS, RS), there is still limited knowledge to help us to understand the observed associations between K and AMF-colonization in our study (but see Benito & González-Guerrero, 2014; Garcia & Zimmermann, 2014). Even though K is a very abundant element of soil composition, it has a low availability due to strong mineral adsorption. In this way, plant-AMF association can improve K uptake, favoring salt and drought stress tolerance in host plants (Benito & González-Guerrero, 2014). To our knowledge, the functional shift in K detected in our study has not been previously observed; however, such change can be associated with K concentration (see Schreiner & Linderman 2005). Accordingly, in DUS, where K was found more limited, we detected the strongest positive effect favoring*R. nudiflora* -AMF association, potentially increasing stress tolerance in *R. nudiflora* individuals from the most extreme urban environment. Considering K in studies focusing in plant-AMF can be fundamental to understand this interaction due its direct and indirect implications mediating plant nutrition (Dibb & Thompson, 1985). Finally, the negative effects of pH detected on P, K, and N are consistent with previous reports (Wiseman & Wells, 2005; Osman, 2013), supporting the idea that the indirect effect of pH can drive, through changes in macro-nutrient availability, the plant-AMF mutualistic interaction.

Despite the fact that several studies have evaluated the effect of urbanization on the plant-AMF association (Egerton-Warburton & Allen, 2000; Wiseman & Wells, 2005; Karliński *et al.* , 2014; Wiseman & Wells, 2005; Buil *et al.* , 2021), few of them have assessed which soil chemical properties predict the intensity of this mutualistic association (Egerton-Warburton & Allen, 2000; Wiseman & Wells,2005; Buil *et al.* , 2021). Egerton-Warburton & Allen (2000) found that vehicular deposition of nitrogen oxides created a eutrophication gradient in which N enrichment reduced AMF root infection in a plant community in Riverside-Perris Plain, Southern California. In the same way, in disturbed landscape soils, Wiseman & Wells (2005) found that AMF-colonization in red maple roots was higher on more acidic soils in Piedmont, South Carolina. They suggest that such increment in AMF infectivity might be due to an indirect effect of acidification, reducing P availability and increasing the benefit of the association with AMF to increase P uptake; however, they did not find statistical differences in P concentrations between developed and undeveloped sites, nor a direct association between P and AMF-colonization. Finally, Buil*et al.* (2021) found that AMF infectivity was negatively associated with subsoil compaction in a gradient of four urban sites with different disturbance levels in Córdoba, Argentina. We believe that recording several soil chemical properties is the key to disentangle complex patterns of association that would help to understand the ecology and evolution of plant-AMF mutualistic interactions. In particular, our preliminary analyses using univariate analyses only detected positive effect of K on AMF-colonization, so the use of SEM was fundamental to disentangle the hidden effects by other macronutrient.

Conclusions

This study is, to our knowledge, the first to explore the ecological effects of urbanization affecting the plant-AMF interaction on an invasive herb. The few studies that have explored the effect of urbanization have focused on the tree-mycorrhizal fungi association (ECM and AMF; e.g. Wiseman & Wells, 2005), and they were mainly concerned about the health of urban trees and ecological services. Recently, Murray-Stoker & Johnson (2021) studied the the white clover (*Trifolium repens*) an invasive herb spread around the globe, and found that the interaction with rhizobium decreased with urbanization due to N enrichment in urban areas in Toronto city. We are convinced that studying invasive and native herbs living in cities and their associations with its microbiota is fundamental to understand the ecology and evolution of the colonization of urban environments. Here, we found that in sidewalks, a deep urban environment which is an inhospitable

environment, P enriched soils play a key role in reducing*R. nudiflora* -AMF mutualistic association.

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Tables

Table 1. Contrast among rural sites (RS), open urban sites (OUS) and deep urban sites (DUS) for rhizosphere soil properties (N, P, K, Ca, Na, pH), plant attributes, and AMF-colonization rates, spore density, and spore diversity of *R. nudiflora.* One-way ANOVA (all soil properties, spore density, and spore diversity) and mixed models (all plant attributes and AMF-colonization rates). Significances between environments were determined using post-hoc Tukey HSD test; different letters between environments indicate significant differences.

	RS	*OUS*	*DUS*	*statistic*	*P-value*	*P-va*
	Soil properties	Soil properties	Soil properties	Soil properties	Soil properties	
N (g/kg)	3.99 ± 0.42^{a}	2.83 ± 0.23^{a}	1.45 ± 0.15^{b}	$\mathbf{F_{2,57} = 27.14}$	**<0.001**	**<0.**
P (mg/kg)	26.50 ± 5.61^{b}	20.33 ± 4.07^{b}	45.13 ± 4.17^{a}	$\mathbf{F_{2,57} = 10.02}$	**<0.001**	**<0.**
K (mmol(+)/kg)	0.74 ± 0.10^{b}	1.73 ± 0.14^{a}	0.14 ± 0.02^{c}	$\mathbf{F_{2,57} = 134.04}$	**<0.001**	**<0.**
Ca (mmol(+)/kg)	77.75 ± 8.28^{b}	181.63 ± 3.94^{a}	25.21 ± 1.16^{c}	$\mathbf{F_{2,57} = 237.92}$	**<0.001**	**<0.**
Na (mmol(+)/kg)	0.18 ± 0.02^{b}	0.89 ± 0.10^{a}	0.08 ± 0.01^{c}	$\mathbf{F_{2,57} = 142.74}$	**<0.001**	**<0.**
pH	7.58 ± 0.02^{a}	7.62 ± 0.03^{a}	7.43 ± 0.03^{b}	$\mathbf{F_{2,57} = 17.54}$	**<0.001**	**<0.**
	Plant attributes	Plant attributes	Plant attributes	Plant attributes	Plant attributes	
Total biomass (g)	3.11 ± 0.25^{a}	1.49 ± 0.10^{b}	2.47 ± 0.19^{a}	$\mathbf{\chi^2_2 = 47.76}$	**<0.001**	**<0.**
Shoot biomass (g)	1.23 ± 0.14^{ab}	0.75 ± 0.06^{b}	1.48 ± 0.14^{a}	$\mathbf{\chi^2_2 = 15.67}$	**<0.001**	**<0.**
Root biomass (g)	1.83 ± 0.18^{a}	0.75 ± 0.06^{b}	1.01 ± 0.08^{b}	$\mathbf{\chi^2_2 = 49.72}$	**<0.001**	**<0.**
Plant height (cm)	19.58 ± 1.24^{ab}	15.36 ± 0.75^{b}	22.10 ± 1.05^{a}	$\mathbf{\chi^2_2 = 6.91}$	**0.0316**	**0.03**
Num. branches	2.15 ± 0.16^{a}	1.68 ± 0.12^{b}	2.41 ± 0.16^{a}	$\mathbf{\chi^2_2 = 19.76}$	**<0.001**	**<0.**
Root volume (cm^3)	5.11 ± 0.34^{a}	2.18 ± 0.18^{c}	3.49 ± 0.28^{b}	$\mathbf{\chi^2_2 = 33.33}$	**<0.001**	**<0.**
Root length (cm)	15.45 ± 0.68^{a}	11.43 ± 0.43^{b}	8.96 ± 0.35^{c}	$\mathbf{\chi^2_2 = 53.99}$	**<0.001**	**<0.**
Num. primary roots	23.51 ± 1.54^{a}	19.05 ± 1.03^{a}	10.47 ± 0.58^{b}	$\mathbf{\chi^2_2 = 89.49}$	**<0.001**	**<0.**
	AMF	AMF	AMF	AMF	AMF	
Colonization (%)	54.49 ± 2.30^{a}	20.67 ± 2.30^{b}	28.77 ± 2.67^{b}	$\mathbf{\chi^2_2 = 31.06}$	**<0.001**	**<0.**
Richness	2.8 ± 1.01^{a}	2.6 ± 0.68^{a}	2.6 ± 1.54^{a}	$\chi^2_2 = 0.1934$	0.9056	0.90
Density (spores /100g)	30.4 ± 5.12^{a}	27.9 ± 5.33^{a}	27.3 ± 4.70^{a}	$F_{2,57} = 1.58$	0.2154	0.21
Diversity (*H'*)	0.73 ± 0.07^{a}	0.54 ± 0.08^{a}	0.62 ± 0.06^{a}	$F_{2,57} = 1.83$	0.1699	0.16

Figure caption

Figure 1. *Ruellia nudiflora* individuals in a) Rural Sites (RS), b) Open Urban Sites (OUS), and c) Deep Urban Sites (DUS) in Mérida City and nearby rural areas. To see the distribution of sampled sites see Fig. S1.

Figure 2. Contrast between urban and rural concentration of a) N, b) P, and c) K, the more relevant macronutrients driving plant-AMF interaction (Heikham *et al.* , 2009; Salvioli di Fossalunga & Novero, 2019). d) AMF-colonization rates (± SE) of *Ruellia nudiflora* by AMF, e) spore density in 100g, and f) diversity Shannon index in *R. nudiflora* rhizospheric soil collected in rural (RS), open urban (OUS), and deep urban (DUS) environments. n.s. indicate no significance, and different letters indicate significant difference ($P < 0.05$).

Figure 3. Biplots of the first two principal components summarizing variation in a) soil properties and b) plant root morphological attributes, for RS (green), OUS (blue), and DUS (orange) environments. Soil PCA (PC_{soil}) (a) used recorded information at site level ($n_{site} = 60$), while root PCA (PC_{root}) (b) depicted included information at plant level ($n_{plant} = 201$).

Figure 4. Association between AMF-colonization rate in *R. nudilfora* roots and the Z-scores for the first principal component ($PC1_{soil}$) from the soil principal component analysis (PCA) in RS (green), OUS (blue), and DUS (orange) environments. Independent linear models were performed for each environment.

Figure 5. Sequential Equation Modeling for soil properties and AMF-colonization rates of *Ruellia nudiflora* . Solid and dashed lines indicate the positive and negative standardized paths, respectively. Path coefficients for RS (green arrows), OUS (blue arrows), and DUS (orange arrows) environments were reported together when detected significantly different by a multigroup test (see methods). Asterisks (*) denote path coefficients that are significantly different from 0. Different letters indicate significant differences between path coefficients. The SEM showed a good overall fit ($\chi^2_2 = 2.661$, $P = 0.264$; CFI = 0.99; RMSEA = 0.074; SRMR = 0.053), as well as a good fit for RS ($\chi^2_2 = 0.883$, $P = 0.643$; CFI = 1.0; RMSEA = 0; SRMR = 0.046), DUS (χ^2_2

= 1.211, P = 0.546; CFI = 1.0; RMSEA = 0; SRMR = 0.046), and OUS (χ^2_2 = 3.287, P =0.193; CFI = 0.00; RMSEA = 0.179; SRMR = 0.092).

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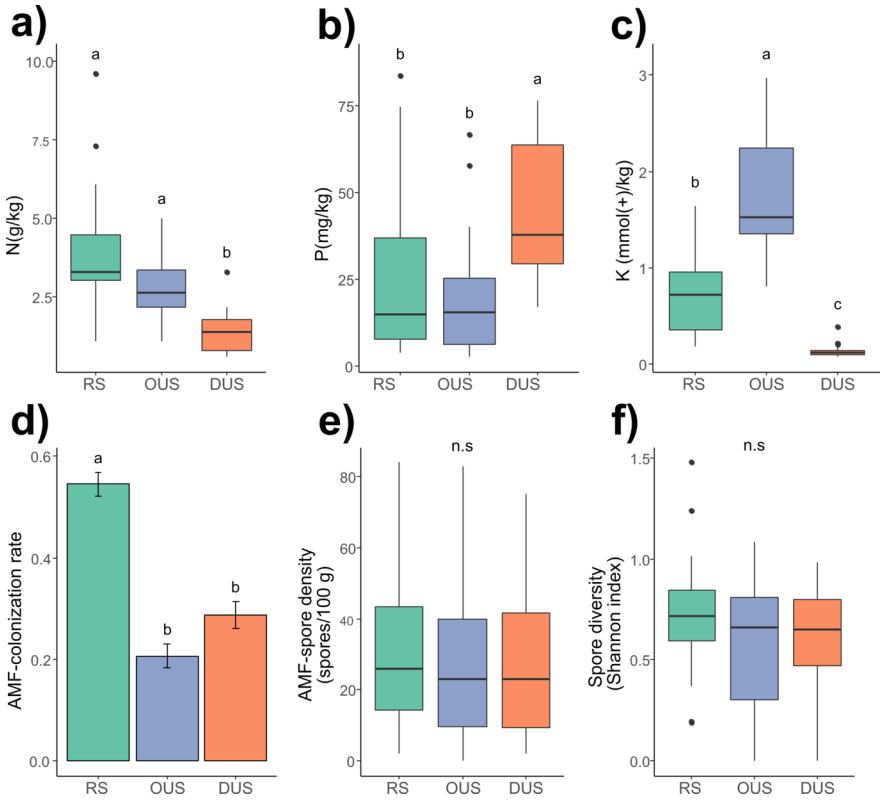

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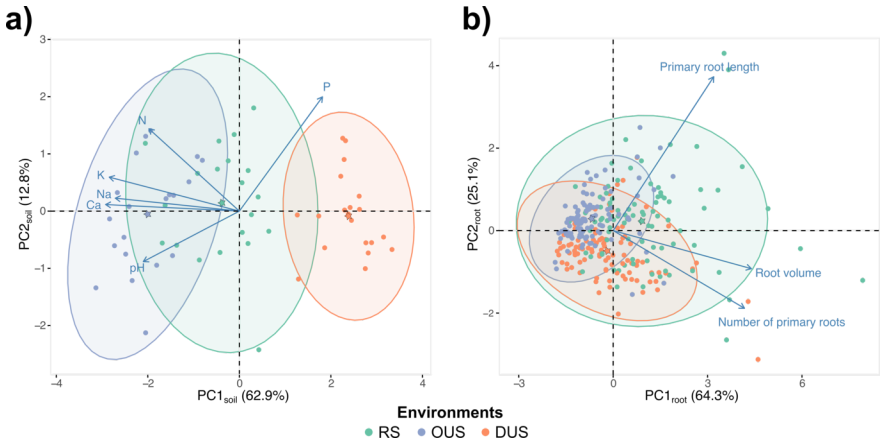

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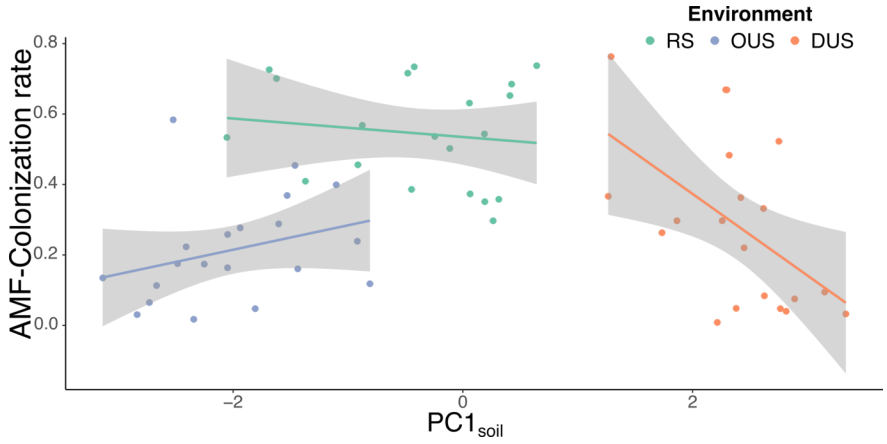

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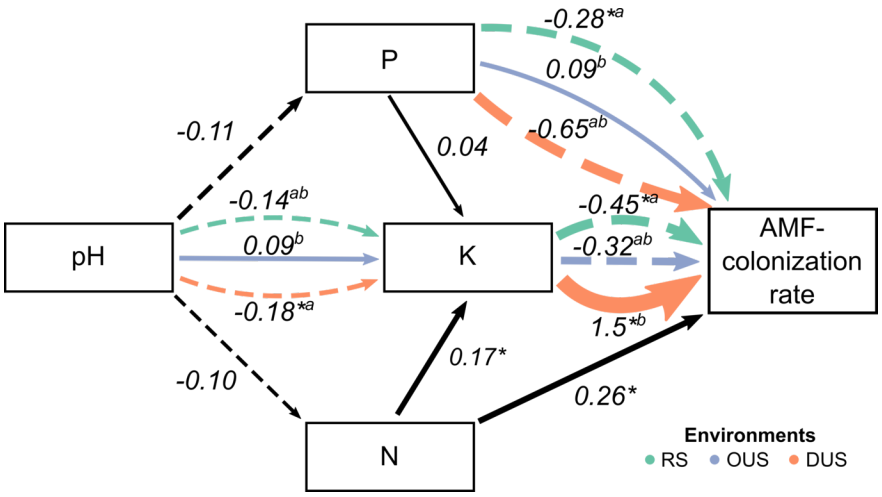

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