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Original Article

Small spatial intervals, big environmental variation: Restinga ecosystems are potential inducers of ecological speciation in the tropical tree *Protium icicariba* (Burseraceae)

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ABSTRACT

Restingas are ecosystems formed by diverse environments and plant populations separated by small spatial intervals. In this sense, we investigated the morphological, anatomical, and physiological characteristics of two populations of *Protium icicariba*, one occurring in a flooded forest and the other in an open *restinga*. Our objective was to determine if the phenotypic responses are related to the local adaptation of each population to its respective habitat and to evaluate a possible incipient case of ecological speciation. We found that individuals in flooded forests are taller and have a higher leaf area, mainly due to the greater availability of water and nutrients. The population in open *restinga* has individuals with higher wood density and smaller and thicker leaves. Sandy, deep, and oligotrophic soils likely induce these characteristics. The reproductive phase of the open *restinga* population is earlier than the flooded forest population, culminating in a certain asynchrony between them. Germination and initial seedling growth performed better when seeds and seedlings were grown in their respective habitats. Our results demonstrate evidence for the development of ecological speciation among *P. icicariba* populations, such as distinct selective pressures and local phenotypic adaptations, pre-zygotic reproductive isolation, and inviability of immigrants.

Keywords: Anatomy; common garden; ecological speciation; morphology; *Protium icicariba*; reproductive phenology; *restinga*

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Introduction

Ecological speciation represents an important mechanism in understanding how species evolve (Nosil, 2012). For the ecological speciation model to develop, the evolution of reproductive isolation must occur between adjacent populations or subsets of a single population by adapting to different environments (Schluter, 2000). Natural selection must be divergent across environments, fixing different advantageous alleles in one location, but which would be disadvantageous alleles in the other (Schluter, 2009). This can occur even among continuous populations and with gene flow if the environments are contrasting (Rundle & Nosil, 2005). Since the environment selects phenotypes, it is suggested that there is often a common genetic basis between the selection of phenotypes and genes involved in the evolution of reproductive isolation (Schluter, 2009). Although the ecological speciation model is well-explored (Nosil *et al.*, 2005), few studies have investigated the real impact of environmental induction (Gilbert & Epel, 2009), especially for tree species (Leigh *et al*., 2004; Stacy *et al.*, 2017).

One potential mechanism for generating reproductive isolation is immigrant infeasibility, which arises from differences in habitat-associated traits (Rundle & Nosil, 2005). Even if crossing occurs between populations or subpopulations adapted to contrasting environments, with seeds dispersed to new areas, reproduction will not be random between native and immigrant individuals. It is expected that individuals will reproduce more frequently with members of the same population compared to members of the immigrant population. This is because migrant individuals have lower survival rates than native individuals, which reduces the possibility of crossbreeding between local and immigrant genotypes (Nosil, 2004). In addition, phenotypes experiencing acclimation to contrasting environments could generate asynchrony between populations' reproductive periods. In this way, gene flow can be totally or partially interrupted between local and migrant populations if each populations have a non-equivalent flowering period (Pfennig *et al*., 2010). Flowering time is often affected by soil water and nutrient availability, leading to accelerated or delayed flowering in different genotypes (Levin, 2009).

Notably, heterogeneous environments can generate disruptive forces and cause intrapopulation divergences (Nosil, 2012). The Atlantic Forest Biome emerges as an important natural laboratory for research related to ecological speciation. It includes many different ecosystems and is classified as a global diversity hotspot (Myers *et al.*, 2000). Among its mosaic of ecosystems, the *restingas*, coastal vegetation types, respond to considerable climatic, edaphic, and ecological variation (Pereira, 2003). They form a set of distinct communities with different forest structures, influenced mainly by variations in the soil's water table level and nutrient composition (Magnago *et al*., 2013; Lourenço Jr *et al*., 2021). Restingas are herbaceous, shrubby, and forest vegetation, which can also be differentiated according to the influence of floods and their floristic composition (Pereira, 2003; Magnago *et al*., 2013). In the periodically flooded forest formation or flooded forest, for example, there are tree-sized individuals colonizing shallow, humid, acidic soil rich in organic matter and with higher concentrations of nutrients (Lourenço Jr *et al*., 2021). In contrast, non-flooded open shrub formations or open *restingas*, even adjacent to forest habitats, have deep, highly sandy soil, are nutrient-poor, and feature shrubby vegetation distributed in thickets (CEPEMAR, 2007). In both habitats, plant populations are habitat specialists and habitat generalists. Among the habitat generalists, *Protium icicariba* (DC.) Marchand var. *icicariba* (Burseraceae) is one of the most common species (Lourenço Jr *et al*., 2020).

Popularly known as almécega or almesca, *P. icicariba* is described as an arboreal, dioecious species endemic to Brazil, with a distribution spanning the states of Bahia, Espírito Santo, and Rio de Janeiro (Daly, 2014). A remarkable characteristic of this species is its high production of aromatic and volatile resins, used by people, among other purposes, as an insect repellent, and in traditional medicine (Rüdiger *et al*., 2007). It is insect-pollinated (Matallana *et al.*, 2005), and its seeds are dispersed by birds (Gomes *et al.*, 2008), lizards, and ants (Pereira *et al*., 2020). It is often listed as one of the most common woody species in the *restinga* communities of Espírito Santo (Ferreira *et al*., 2010; Ferreira & Silva, 2014; Lourenço Jr *et al*., 2020). Kurtz *et al.* (2013) state that *P. icicariba* can inhabit open *restinga* and flooded forest areas. Such adaptive ability suggests that this species is a habitat generalist.

Our objectives in this study were: (i) to investigate the properties of the soils of the flooded forest habitat and in the open *restinga* habitat, (ii) to investigate whether populations of *P. icicariba*, distributed in the two habitats, exhibit functional differences in morphological and anatomical traits in their leaves and branches; (iii) to analyze the synchrony between the reproductive phenology of the two populations and, (iv) utilizing germination analysis and the initial growth of seedlings, to verify the viability of immigrants across the two habitat types. We do not intend to test for the factors causing lineage divergence directly. Instead, we aim to document the conditions consistent with ecological speciation. Our predictions are: (I) morphological and anatomical differences of the two populations in each habitat will be mainly associated with differences in nutrient concentrations and soil water availability of each habitat; (II) there will be asynchrony in the reproductive phenology and (III) sub-optimal performance in the development and survival of immigrants.

Materials and Methods

Study area

The study area is in Paulo Cesar Vinha State Park, at Km 37.5 of the ES-060 highway (*'Rodovia do Sol'*), (20° 35'25" S; 40° 25'24" W), municipality of Guarapari, state of Espírito Santo, Brazil (Fig. 1). According to the Köppen-Geiger climate classification system, the climate in the region is Aw, tropical hot and humid with a rainy season (summer) and a dry season (winter). The average annual rainfall for the capital Vitória is 1,276 mm, and the average annual temperature is 24.2 °C (INMET, 2022).

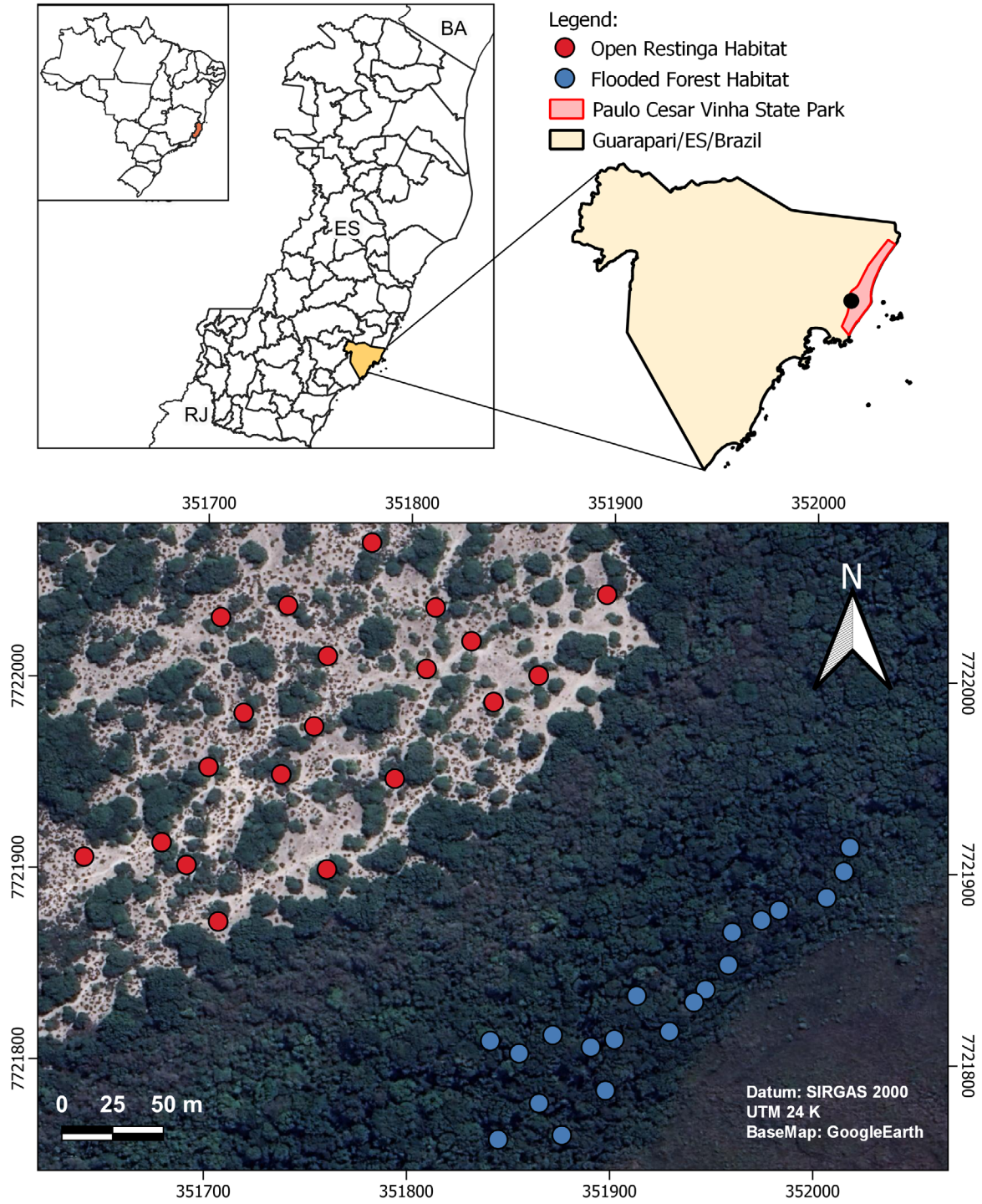

Figure 1. Study area in Paulo Cesar Vinha State Park (PEPCV). Individuals of *P. icicariba* in the open *restinga* habitat are represented by red spheres. Individuals of *P. icicariba* in the flooded forest habitat are represented by blue spheres.

Two *restinga* habitats were selected, on average 150 m apart. The first habitat, open *restinga*, is situated between the flooded forest and the ES-060 highway (Fig. 1). The vegetation cover consists of thickets of low stature and sparse populations of herbaceous species colonizing strips of white sand. The soil is predominantly quartz with a sandy texture and a deep-water table (CEPEMAR, 2007). The second is a flooded forest, and it is in a relief depression area between a flooded herbaceous habitat and a non-flooded forest habitat. This forest zone has a flat topography, visually dark soil, and a characteristic odor of decaying organic matter. Compared to other forest areas of the same *restinga*, it has a higher accumulation of organic matter, acidity, aluminum and salt concentrations, poor drainage, and a shallow water table (CEPEMAR, 2007; Magnago *et al*., 2013; Lourenço Jr *et al.*, 2020).

Climatic data and soil analysis

We recorded temperature and precipitation data from June 2018 to June 2021 during the experimental period to compare them with historical averages. The climatic variables were obtained from the automated weather station A634 in the municipality of Vila Velha-ES (INMET, 2022). During the experimental period, there were no significant variations in air temperature and precipitation in relation to historical data for the region (Figure S1).

We collected soil samples in each tree or shrub's canopy projection. Using a drill, we extracted four single samples at 0 to 20 cm depth. Then, we homogenized the four sub-samples and stored them in plastic bags for forwarding to the laboratory. We performed chemical, physical, and organic matter analyses (Table S1) using the analytical method used by Teixeira *et al.* (2017).

Determination of Sample Populations, Height, and Stem Circumference

We sampled 20 individuals of *P. icicariba* from the open *restinga* habitat and 20 individuals from the flooded forest habitat (Fig. 1). We selected adult individuals with a stem diameter at height (DBH) ≥ 5 cm, respecting a minimum spacing of 15 meters between individuals from the same sampled population. For the shrub population, the stem diameter was determined at ground level due to the small length of the aerial stem and the beginning of branching at ground level (Fig. 2C). Height and circumference were determined using a tape measure. All subjects were georeferenced and marked with numbered aluminum plates for monitoring phenological events.

Morphological and anatomical analyses

For all morphological and anatomical analyses of the leaf and stem, we used a sample size of 20 individuals per population. We collected all samples on the same day and analyzed them in the laboratory. Morphological data were obtained with fresh samples. Three fully expanded leaves exposed to the sun per individual were collected along the canopy. We analyzed the number of leaflets per leaf by adding all leaflets on each leaf and calculating the average number. The blade thickness was determined with the aid of a digital caliper in three leaflets per leaf, the fresh mass utilizing a precision scale, and the leaf area with the aid of the leaf area meter (LICOR-3100C Area Meter). Leaf dry mass was obtained by drying the leaves in an oven at 60°C (Leucadema-LM300) for 7 days. The specific leaf area was obtained by calculating the ratio between the leaf area and its dry mass ($cm^2\ g^{-1}$).

Three leaflets from each individual (one per leaf) were fixed in FAA 70 (37% formaldehyde, glacial acetic acid, 70% ethanol, in a 5:5:90 ratio) (Johansen, 1940) and stored in 70% ethyl alcohol. Cross-sections were performed freehand, and after double staining with safrablau (Kraus & Arduin, 1997), they were mounted in pure glycerin. In the interveinal region, quantitative analyses were performed by measuring the thickness of the adaxial and abaxial cuticles, the epidermal cells of the adaxial and abaxial faces, and the palisade and spongy parenchyma. We measured each variable using one leaflet per individual, using a slice to perform twenty thickness measurements per anatomical structure. Then, we calculated the meaning of the variable per individual and population. We determined the stomatal density (number mm^{-2}) and the diameter of the stomata utilizing the epidermal impression technique using a drop of universal instant adhesive (Tek Bond®) on the abaxial face of the leaflets on a glass slide to obtain the impression of the epidermis for analysis. The stomatal length was determined based on the average length of the guard cells (one leaflet per individual, 40 stomata per leaflet). All slides were analyzed using image analysis software (Nikon NIS–Elements, Tec. Behavior, Tokyo, Japan) and documented using a Nikon model photomicroscope (Eclipse 50i, Japan).

The sampling of branch segments was standardized concerning their position along the tree (first basal branch), their position along the branch (~1 m from the branch tip), their diameter (1.10 ± 0.37 cm, without the bark), and their maturity. To determine the wood density, the samples were hydrated for 12 hours, and the wood volume was calculated according to the water displacement method (Chave, 2006) with the support of a precision balance. Then, the samples were dried in an oven at 60° C until constant weight. The wood density was determined by dividing the dry mass by its volume.

From samples of branches stored in 70% ethyl alcohol, sections of approximately 15 to 20 μm thick cross-sections were obtained with a Jung microtome for wood. The sections were stained with 1% aqueous safranin and 1% alcian blue (1:9) (Kraus & Arduin, 1997). Histological slides were permanently mounted with stained glass varnish. We determined all samples's vessel frequency and diameter from an image using TSview software. The vessels present partially or totally in 1 mm^2 were counted, and their diameter was determined from the average of two cross-sectional measurements in each cell.

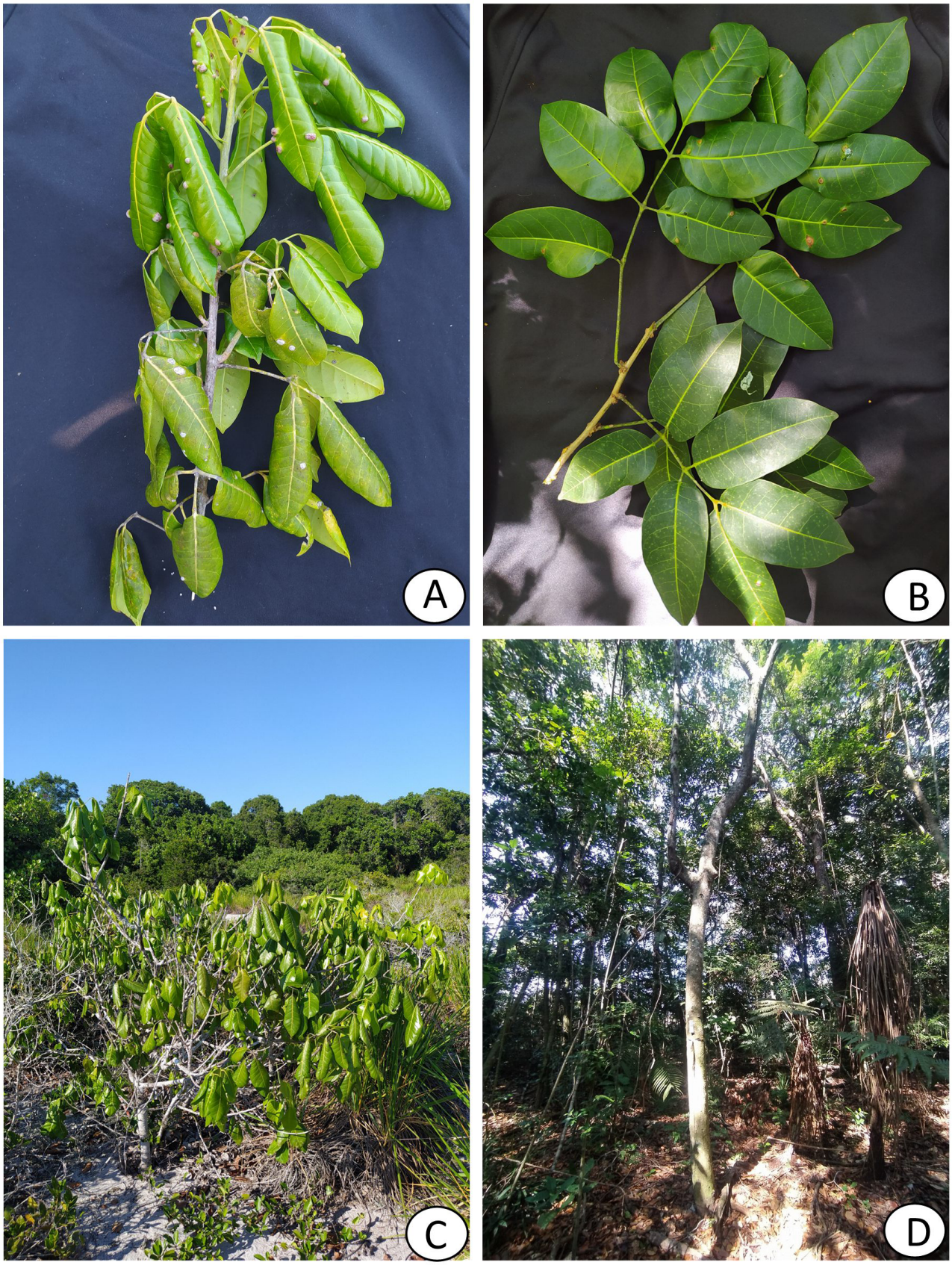

Figure 2. Individuals and branches of *P. icicariba*. (A) and (C) – Individual of the open *restinga* population; (B) and (D) – Individual of the flooded forest population.

Reproductive phenology

We recorded reproductive phenological events biweekly for 24 months (September 2018 to August 2020) in 20 individuals per population. We observed flowering and fruiting with the aid of binoculars and a camera. The flowering and fruiting times were quantified in terms of their activity index, which indicates the percentage of individuals in the population manifesting the phenological event and the synchronous comparison of these events between populations (Bencke & Morellato, 2002). Flowering was defined as the period in which individuals presented buds until the anthesis of the last flowers, and fruiting was defined as the period from the appearance of the first fruits until ripening.

Germination

Fruits were harvested during the dispersal period from ten individuals in the open *restinga* and five in the flooded forest. Subsequently, the fruits were transferred for processing in the laboratory to remove the epicarp and mesocarp. The seeds were then placed in fine mesh polyester envelopes and buried in the soil to a depth of 2–3 cm, following (with modifications) the methods adopted by Porceddu *et al.* (2013) and Sarmiento *et al.* (2017). We filled eight envelopes with seeds from open habitats and eight envelopes with seeds from flooded forest habitat individuals, each containing 30 seeds (Figure S2). We selected four germination sites in each habitat to increase the experimental area. Each site was located beneath the canopy of a *P. icicariba* tree, containing one envelope with seeds collected from the same habitat and one envelope with immigrant seeds collected from the alternative habitat. The envelopes were exhumed at 20-day intervals, with a maximum duration of 80 days, according to the results of Zamith & Scarano (2004). At each exhumation, the germinated seeds were counted to determine the percentage and stage of germination. We considered germination to be the protrusion of the radicle or the emission of shoots by the seedling.

Survival and emission of new leaves

Seedlings measuring 20 and 30 centimeters were cultivated in common gardens from September 2020 to March 2021. We extracted 40 seedlings from the open *restinga* and 40 from the flooded forest, keeping part of the soil in the rhizosphere. Afterward, 50% of the individuals were replanted in their environment of occurrence (native) and 50% in the opposite environment to their occurrence (immigrant). In each habitat, four planting sites were established. The seedlings were replanted circularly and in pairs at each site, with five immigrant seedlings and five local ones (Figure S3). We watered the seedlings at the time of planting and, in the absence of precipitation, at three-day intervals during the first month. All seedlings were marked with wire and aluminum tags.

We monitored all transplanted seedlings from planting through the six-month study period, recording survival rates and measuring new leaf production monthly. Only fully expanded leaves were included in the leaf count.

Statistical analysis

We evaluated the normality of the data using the Shapiro-Wilk test. Data sets that showed normal distribution were submitted to the Student's t-test for independent samples ($P \leq 0.05$). The means of variables that were not normally distributed were analyzed using the Mann-Whitney test for independent samples ($P \leq 0.05$). To better understand the morphological and anatomical variations between populations, we used principal component analysis (PCA). All statistical analyses were conducted in R version 4.1.0 (R Core Team, 2021) using exclusively the base stats package.

Results

Soil analysis

Soil data showed considerable chemical and physical significant differences between *restinga* habitats. The flooded forest, in relation to the open *restinga*, presents higher relative concentrations of potassium (64.5%), sulfur (70.4%), calcium (70.4%), magnesium (59.2%), aluminum (285.4%), potential acidity - H+Al (235.9%), organic matter (143.3%), iron (119.2%), zinc (158.7%), boron (31.6%), sodium (134.23%), fine sand (117.9%), silt (1,841.3%) and clay (169.0%) (Table 1). On the other hand, the soil of the open *restinga* presented higher averages of pH (9.2%) and total sand (57.2%), with a higher proportion of coarse sand (97.1%). As for the textural classification, the soil of the flooded forest was defined as loam-silty, while the soil of the open *restinga* was classified as sand (Table 1).

Morphological and anatomical variables

In the flooded forest habitat, individuals are significantly larger in height (221.1%), leaf area (124.5%), specific leaf area (38.1%), and leaflets per leaf (47.2%) compared to those in the open *restinga* habitat (Table 2). The individuals in the open *restinga* have branched stems with larger diameters (35.4%). Their leaves are composed of a thicker blade (12.5%), and their wood is denser (8.6%) (Table 2).

Populations did not show significant differences in woody anatomy (Table 3). However, the leaf traits indicated that the open *restinga* population presents all the highest relative averages (Table 3). These are stomatal length (19.8%), abaxial (46.3%) and adaxial (65.9%) cuticles, abaxial (14%) and adaxial (34.9%) epidermis, in addition to spongy parenchyma (11.5%) and palisade (30.8%) (Table 3, Figure S4).

Table 1. Comparative soil analysis between two habitats, open restinga and flooded forest of Paulo Cesar Vinha State Park, in Guarapari, in Espírito Santo, Brazil.

	Characteristics	Habitat		p-value	CV (%)
		Open restinga	Flooded Forest		
Chemical Analysis	Phosphorus (P)[1]	3.65	5.75	0.0579	72.26
	Potassium (K)[1]	22.10	36.35*	0.0035	49.52
	Sulfur (S)[1]	5.75	9.80*	<0.0001	35.59
	Calcium (Ca)[2]	0.88	1.50*	0.0029	52.34
	Magnesium (Mg)[2]	0.27	0.43*	0.0065	48.95
	Aluminum (Al)[2]	0.96	3.70*	<0.0001	32.70
	H+Al[2]	13.41	45.04*	<0.0001	57.75
	pH	4.64*	4.25	0.0001	6.38
	Organic matter[3]	6.53	15.89*	<0.0001	32.93
	Iron (Fe)[4]	27.3	59.85*	0.0005	61.69
	Zinc (Zn)[4]	0.63	1.63*	<0.0001	59.70
	Copper (Cu)[4]	0.24	0.29	0.4026	70.49
	Manganese (Mn)[4]	1.20	1.35	0.3002	35.42
	Boron (B)[4]	0.76	1.00*	<0.0001	17.88
	Sodium (Na)[4]	17.65	41.35*	<0.0001	46.78
Physical Analysis	Course sand[5] (2 to 0.20 mm)	927.20*	530.80	<0.0001	16.60
	Fine sand[5] (0.2 to 0.05mm)	27.60	76.70*	0.0001	70.62
	Total sand[5] (2 to 0.05mm)	954.80*	607.50	<0.0001	16.59
	Silt[5] (0.05 to 0.002mm)	16,20	314,50*	<0,0001	73,47
	Clay[5] (> 0.002mm)	29,00	78,00*	<0,0001	33,63
	Textural classification	Sand	Silty-loam	—	—

The asterisk (*) indicates a significant difference between the mean of open restinga and flooded forest by Student's t-test or Mann–Whitney at a 5% significance level (n = 20). In which: [1]mg dm^{-3}, [2]cmolc dm^{-3}, [3]dag dm^{-3}, [4]mg cm^{-3} and [5]g kg^{-1}.

Table 2. Comparative analysis of morphological attributes between two populations of *P. icicariba* occurring in open restinga habitat and flooded forest habitat.

Variables	Open restinga	Flooded forest	p-value	CV (%)
Height (m)	2.51	8.06*	<0.0001	20.83
Stem diameter (cm)	17.11*	12.64	0.0398	44.67
Wood density (g cm^{-3})	0.63*	0.58	0.0082	8.63
Leaf area (cm^2)	94.74	212.73*	<0.0001	24.63
Leaflets per leaf (n)	3.54	5.21*	<0.0001	15.12
Limbo thickness (mm)	0.27*	0.24	0.0093	15.77
Specific leaf area (cm^2 g^{-1})	69,93	96,59*	<0,0001	12,64

The asterisk (*) indicates a significant difference between the mean of open restinga population and flooded forest population by Student's t test or Mann–Whitney at a 5% significance level (n = 20).

Table 3. Comparative analysis of anatomical attributes between two populations of *P. icicariba* occurring in open restinga habitat and flooded forest habitat.

	Characteristics	Open restinga	Flooded forest	p-value	CV (%)
Thickness	Abaxial cuticle[1]	2.59*	1.77	0.0019	35.5
	Adaxial cuticle[1]	4.53*	2.75	<0.0001	31.9
	Abaxial epidermis[1]	6.82*	5.98	0.0193	17.0
	Adaxial epidermis[1]	11.53*	8.55	0.0019	28.0
	Palisade parenchyma[1]	77.12*	58.95	<0.0001	13.9
	Spongy parenchyma[1]	119.49*	107.16	0.0067	12.0
	Length stomatal[1]	70.27*	58.65	0.0009	15.8
	Vessels diameter [1]	57.65	57.60	0.9898	18.1
	Stomatal density [2]	31.4	28.8	0.2043	21.2
	Vessels density [2]	81.0	69.8	0.0705	25.4

The asterisk (*) indicates a significant difference between the average open restinga population and the flooded forest population by Student's t-test or Mann–Whitney at a 5% significance level (n = 20). Where: [1]µm, [2]n mm^{-2}.

The first principal components (PC1 and PC2) explained 68.9% of the variation between *P. icicariba* populations among morphological characteristics (Fig. 3A) while the same components explained a variation of 55% for the anatomical features. The distribution of the values of all individuals's morphological and anatomical variables within the multivariable space highlights that the individuals of the open *restinga* are displaced and grouped in a region opposite to that occupied by the flooded forest. Individuals from open *restinga* were characterized by distinct stem diameter, wood density, blade thickness, and almost all anatomical variables. Meanwhile, the individuals in the flooded forest were associated with greater height, leaf area, fresh mass, specific leaf area, and the number of leaflets per leaf.

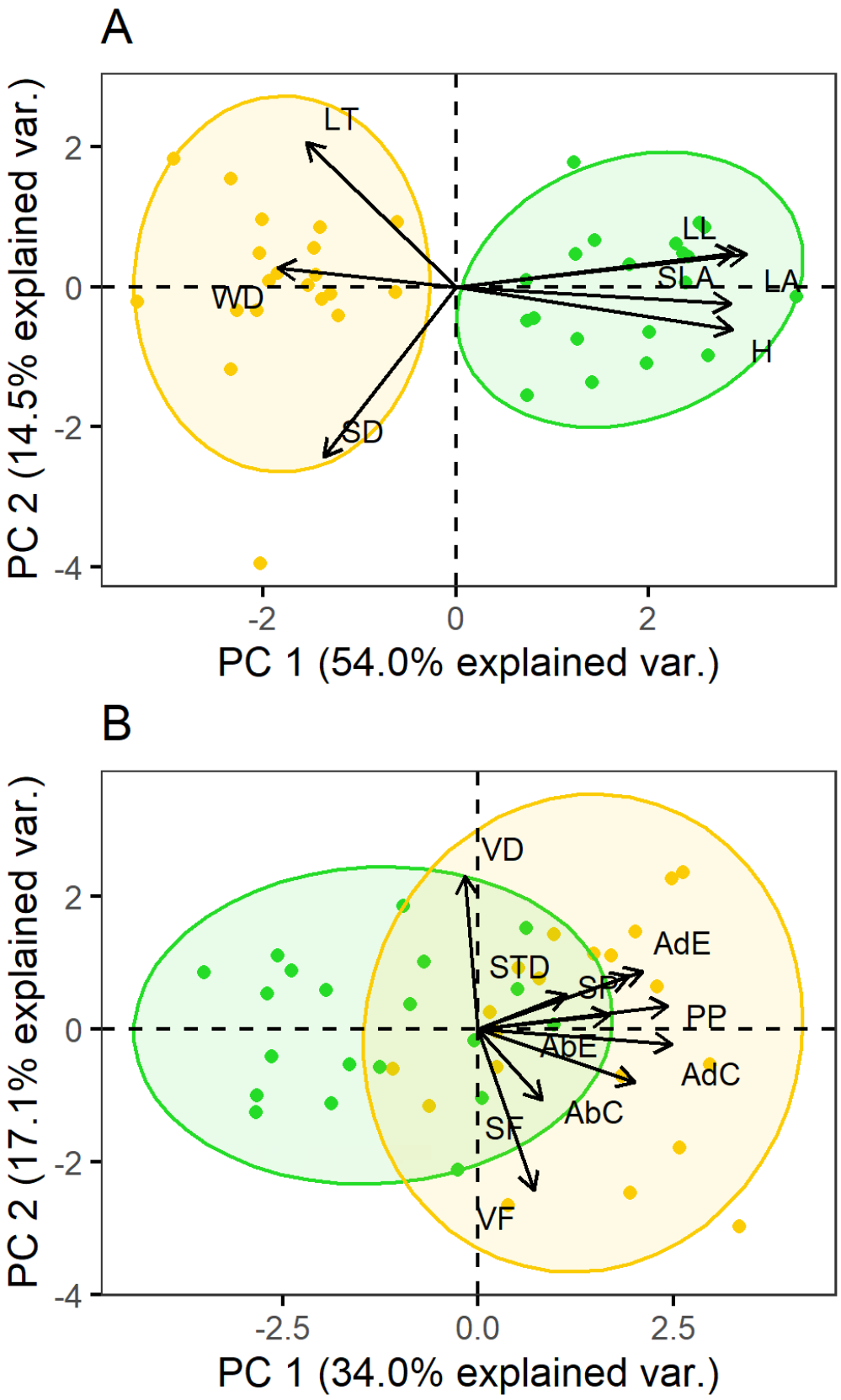

Figure 3. Principal component analysis (PCA) for quantitative morphological and anatomical variables of two populations of *P. icicariba*, open *restinga* (yellow circles) and flooded forest (green circles). H = height; LA = total leaf area; SLA = specific leaf area; LL = number of leaflets per leaf; STD = stem diameter; WD = wood density; LT = limb thickness; VF = vessel frequency; VD = vessel diameter; SF = stomatal frequency; SD = stomatal diameter; AdC = adaxial cuticle; AbC = abaxial cuticle; AdE = adaxial epidermis; AbE = abaxial epidermis; PP = palisade parenchyma; SP = spongy parenchyma.

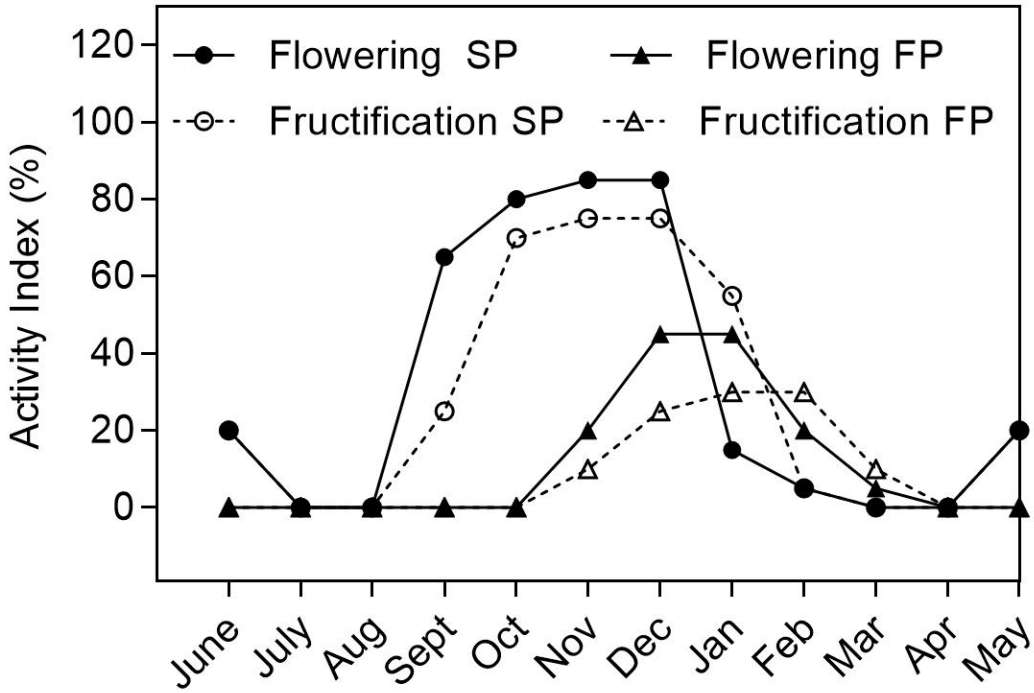

Figure 4. Reproductive activity index of two populations of *P. icicariba* distributed in the open *restinga* and flooded forest in Paulo Cesar Vinha State Park, municipality of Guarapari, state of Espírito Santo, Brazil. SP = shrub population; FP = forest population.

Reproductive phenology

The flowering peak of the open *restinga* population is concentrated between September and December, during which up to 85% of the individuals flower (Fig. 4). Fruiting in this population is also concentrated between the months of October and January, with fruits found in 75% of the individuals sampled. The percentage of individuals from the flooded forest population that presented inflorescences and fruits was limited to 45% and 35%, respectively. The flowering peaks in the forest population are concentrated between December and January, with some individuals flowering until March. Fruiting occurs between the months of November to March, with peaks between December and February.

Germination, survival, and growth

In general, *P. icicariba* seeds germinated during the period of 80 days. However, they presented low germination rates (Figs. 5A, 5B). The flooded forest had a significantly higher average germination rate (32.1%) than the open *restinga* (16.7%) when considering native and immigrant seeds. However, native seeds consistently showed higher germination rates, regardless of the area. The first thirty days after transplanting had the highest seedling mortality rates in both plant habitats (Figs. 5C, 5D). Among the eighty seedlings, nineteen died in the first month (23.8%). Mortality was significantly higher in the open *restinga* habitats compared to the flooded forest. In the open *restinga* habitat, the immigrant seedlings showed a significantly higher number of losses (75%), with only five individuals left alive compared to eleven individuals from the native population at the end of the experiment. In the flooded forest, mortality was lower during the same period, with little difference between native and immigrant seedlings. In this environment, fourteen native seedlings survived after six months of the experiment, compared to twelve seedlings from the immigrant population.

In the open *restinga* habitat, the native seedlings produced a significantly higher number of leaves for 180 days (Fig. 5E). On average, native seedlings produced 4.8 leaves, while immigrant seedlings produced only 2.4 new leaves. The transplanted seedlings in the flooded forest habitat showed the same pattern (Fig. 5F), with native seedlings producing a significantly higher number of leaves. On average, the native ones produced 6.8 leaves, while the immigrant seedlings produced only 3.6 new leaves.

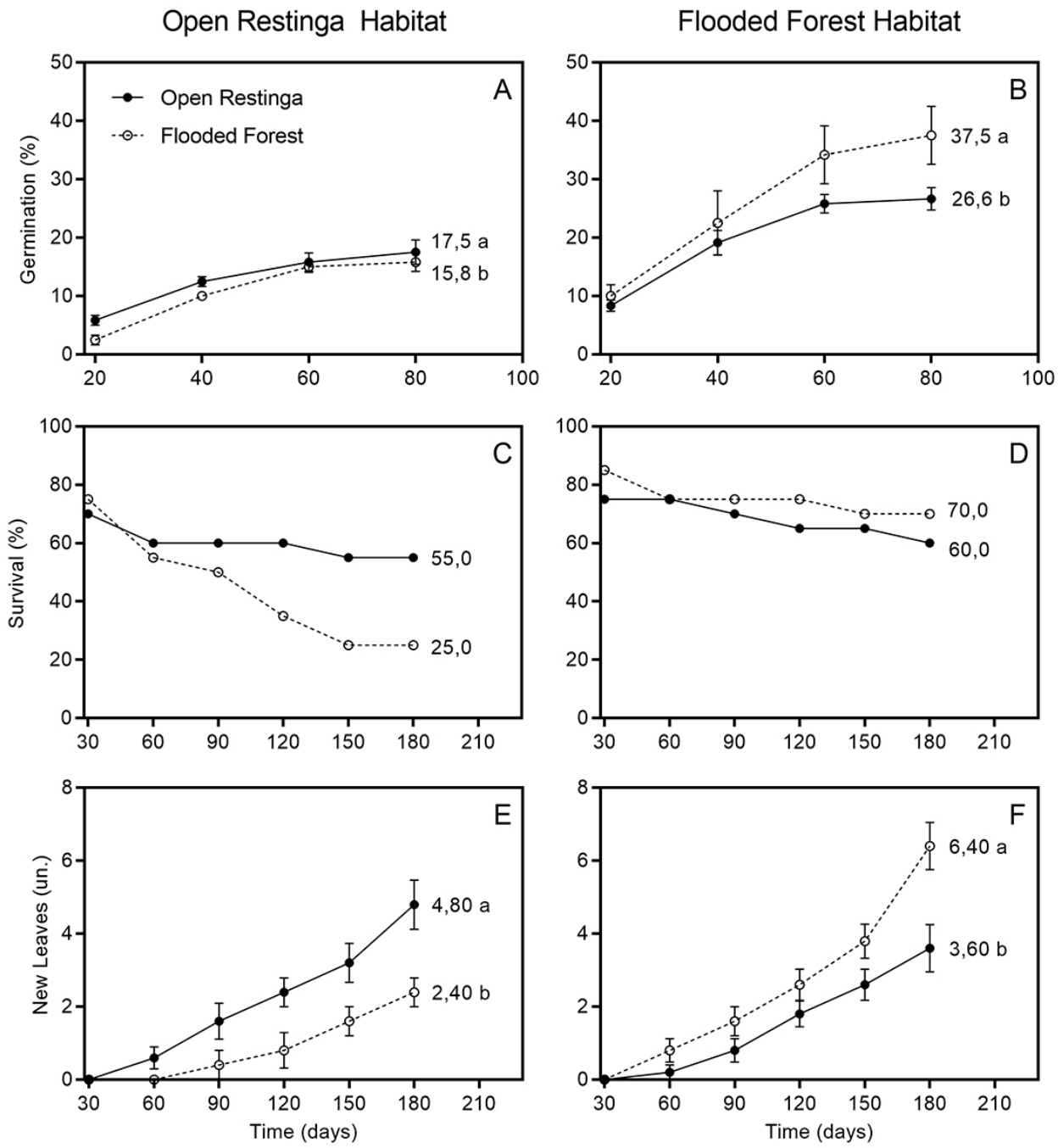

Figure 5. Germination (A and B), issue of new leaves (C and D), and survival (E and F) of *P. icicariba* seedlings in open *restinga* and flooded forest habitat. The solid line represents seeds from the open *restinga* population and the dashed lines represent seeds from the flooded forest population. The letters compare the means of individuals from each population, Student's t-test or Mann–Whitney ($P \leq 0.05$).

Discussion

Environmental correlates with morphological and anatomical traits

As expected, the flooded forest habitat presents greater availability of macro and micronutrients essential for plant development. This is because, in deeper soils, such as those on the open *restinga*, there is greater nutrient leaching (Lourenço Jr & Cuzzuol, 2009; Magnago *et al*., 2010), mainly due to sandy soil texture (Rodrigues *et al*., 2013). This nutritional difference, associated with the variation in the depth of the water table and the water retention capacity in the soil, largely explains the specific morphological and anatomical adaptations of each population, corroborating the first hypothesis. We highlight that selecting phenotype combined with significant environmental differences is the first condition for developing ecological speciation (West-Eberhard, 2003).

The forest environment has a better water supply due to the higher concentration of clay and organic matter, but mainly due to the reduced depth of the water table. This condition, combined with higher concentrations of essential nutrients, certainly contributes to the presence of taller individuals in an environment with greater competition for resources (Kunstler *et al*., 2016). Investing in growth, for example, would be necessary to reach the canopy in an environment of competition for light. On the other hand, the higher levels of salts and high concentrations of aluminum, together with the low pH, could limit the distribution of species (Magnago *et al*., 2013), acting as filters in the functional and taxonomic composition of the plant community (Poorter *et al*., 2009; Lourenço Jr *et al*., 2020). However, individuals from the forest population could circumvent this limitation using salt tolerance and exclusion mechanisms (Poorter *et al*., 2009) or having exclusion and tolerance to Al available in acidic soils (Schmitt *et al*., 2016).

The higher number of leaflets per leaf and leaf fresh mass reinforce the development of larger leaves in the flooded forest habitat, and the former can become an important taxonomic classification character. This is because the availability of water and nutrients allows investment in expanding the leaf area (Kozuka *et al*., 2005). In addition, larger and thinner leaves in forest individuals can facilitate light penetration throughout the palisade parenchyma and optimize photosynthetic activity (Da Silveira *et al*., 2015). The shaded environment also stimulates an increase in leaf area and biomass accumulation to optimize the interception of the low light penetrating the canopy (Niinemets, 2010; Poorter *et al*., 2012), a characteristic reflected in higher rates of specific leaf area. Due to the more excellent supply of water, individuals in the flooded forest do not need to have greater stocks of water in the spongy parenchyma, influencing a smaller blade thickness and, consequently, an increase in the biomass concentration and the specific leaf area index (Da Silveira *et al*., 2015).

On the other hand, the open *restinga* is an environment with xeric characteristics. The soil in the open *restinga* presents characteristics observed in soils that retain low amounts of water (Cooper *et al*., 2017). It is mainly composed of coarse sand, with low concentrations of clay and organic matter and a deeper water table than that observed in the flooded forest (Lourenço Jr *et al*., 2021). For these reasons, *P. icicariba* individuals have common strategies of plants adapted to drought, such as investing in lower height, leaf area, and specific leaf area (Cornwell & Ackerly, 2009; Katabuchi *et al*., 2012), production of thick leaves and scleromorphic tissues (Wright *et al*., 2004), and greater wood density (Sur *et al*., 2018). The smaller specific leaf area is related to oligotrophic and low-humidity soils, with the development of scleromorphic leaves (Turner, 1994), as observed in some woody plants that grow in sandbank soils (Melo Jr & Boeger, 2016). This characteristic can promote longer leaf longevity in a resource-poor environment. Thick leaves prevent oxidative damage to the photosynthetic apparatus, increasing resistance to irradiance penetration (Rossatto & Kolb, 2010; Todorovski *et al*., 2015). With thick, spongy parenchyma, the plant has greater water accumulation in the leaf, and the smaller leaf area reduces water loss through transpiration (Boeger & Gluzezak, 2006).

Although not analyzed in this research, the leaves of shrub individuals were commonly found to be rolled up (Fig. 2A). This attribute is known to reduce light interception, transpiration, and leaf dehydration (Kadioglu *et al*., 2012). Thus, leaf rolling in shrubs may be related to the indifference to the size and frequency of stomata among populations, as we expected the shrub population to have smaller and more numerous stomata. Leaves with smaller and more numerous stomata are more efficient in water use in xeric environments (Abrams *et al*., 1994; Rossatto & Kolb, 2010). In this case, leaf rolling could mitigate xeric effects by altering the microclimate on the leaf surface, regulating water loss (Matthews *et al*., 1990).

Some results appear to be more specific and would not be expected based on the large differences in height of the individuals. Despite being considerably smaller, shrubs have a larger stem diameter. However, this characteristic could be explained by the species presenting vegetative propagation and underground stems (Ferreira *et al*., 2010). Therefore, we consider nearby stems belonging to the same individual, which considerably increase their diameter together. However, the higher wood density observed in these individuals aligns with our expectations. This is a common strategy in trees subjected to xeric environments, as it provides higher survival probabilities by avoiding cavitation (Chave *et al*., 2009). However, the higher density of wood is not accompanied by a lower diameter or frequency of vessels in the shrub population. Alternatively, higher wood density may be related to fibers with thicker walls (Pratt *et al*., 2007) and, consequently, higher concentrations of structural polymers.

Reproductive phenology

The differences observed between the flowering peaks support our second hypothesis, as there is asynchrony between the reproductive periods of populations of *P. icicariba* among the investigated habitats. Reproductive phenology then favors crosses between individuals of the same population, allowing for the establishment of genetic variations related to their adaptive phenotypic traits. In this model, partial reproductive asynchrony is a strong assumption for ecological speciation under disruptive selection and gene flow (Fry, 2003; Kopp *et al.*, 2018). That is, if there are variable adaptive phenotypes between populations, they can be maintained preferentially through local crossbreeding. The first individuals to flower will tend to interbreed with other "early" ones, and the late ones with other "late" ones (Weis *et al.*, 2005). In this case, the chemical and physical composition of the soil could induce inconsistent reproductive cycles between continuous populations. Brady *et al.* (2005) suggest that adapting plants to contrasting edaphic conditions is important for isolating varieties in reproductive character. In *P. subserratum* growing parapatrically throughout western Amazonia, genetic differentiation and morphological variation among populations were correlated with soil type (Misiewicz & Fine, 2014). Contrary to our findings, populations of *P. subserratum* growing in different soil types did not exhibit asynchrony in flowering time; however, five potential barriers to reproduction were identified, including ecogeographic isolation, phenological differences, differences in pollinator assemblages, differential pollen adhesion, and low levels of hybrid seed development. However, ecogeographic isolation accounted for most of the observed isolation among ecotypes (Misiewicz *et al.*, 2020).

Considering that the flowering peak of the shrub population is earlier in relation to the forest population, we assume this may be a drought avoidance strategy. When the species disperses its seeds during the rainy season, it guarantees a better germination rate in the shrub environment. Otherwise, germination would be compromised in the drier months because the sandy soil of this habitat retains less moisture and has greater exposure to sunlight. On the other hand, the dispersion of seeds from the forest population in their natural environment can occur at the end of the rainy season, as the soil characteristics in this habitat must guarantee sufficient moisture, even during the dry season. In addition, not dispersing the seeds during the rainiest period may be a strategy so that their seeds are not subjected to flooded summer environments, a condition that would make the survival of the embryos unviable. This may occur due to a predictability mechanism that matrices develop in their habitats (Fenner, 2017; Bhatt *et al.*, 2020), where plants reproduce under more favorable conditions.

Immigrant performance

The sub-optimal performance of immigrants corresponds to our third hypothesis because even if germination and seedling development are possible, they present inferior performance in relation to native individuals. Therefore, highly antagonistic environments, such as open *restinga* and flooded forest habitats of *restinga*, hinder the generalist habit of species in the ecosystem. The flooded forest offers the best conditions for the initial establishment of the species since, in general, germination, survival, and the emission of new leaves are significantly better in this environment. However, it is generally noted that seeds or seedlings submitted to their native environment outperform immigrants. These observations follow the same pattern found in a similar study model with *Metrosideros polymorpha* (Myrtaceae), where seeds generally germinated at higher rates in more humid environments, and young individuals generally performed better in their places of origin (Barton *et al.*, 2020). In another study, using a reciprocal transplant experiment with *Protium subserratum* (Burseraceae), Fine *et al.* (2013) observed that plants in *terra firme* (non-flooded) forests exhibited significantly greater height and leaf growth than the white-sand forest populations in both soil types.

Some characteristics of the forest environment may have disadvantaged the germination performance of the shrub population, indicating that local seeds are better adapted to these conditions. The higher concentration of salts, for example, can reduce the osmotic potential of the soil, making it difficult for the seed to absorb water (Munns, 2002), and soil acidity can reduce germination rates (Belmehdi *et al.*, 2018). In contrast, in the open *restinga* environment, the seeds had to deal with low humidity, higher temperatures, and light exposure. Water deficiency causes the soil matric potential to be more negative, making it difficult for the seed to absorb water (Guedes *et al.*, 2013), and the interaction between light and temperature influences the reactions that regulate the metabolism necessary to initiate germination (Baskin & Baskin, 2014). Therefore, due to the specific performance of seeds in their environment of origin, it is not ruled out that there may be interpopulation genetic variation that meets the requirements for germination in each environment (Murru *et al.*, 2017). Or, as already mentioned, during seed production and maturation, parental individuals can perceive and adjust their phenotypes according to environmental requirements and optimize embryo development (Gutterman, 2000).

The environmental conditions of the open *restinga* habitat cause greater difficulties in establishing *P. icicariba* in the initial phase of seedling growth. Water stress, for example, can affect the production of new leaves (Nascimento *et al.*, 2011). The reduced nitrogen supply can direct resource allocation for root development (Poorter *et al.*, 2012). However, because native seedlings perform better on islands of vegetation in the open *restinga* habitat, it suggests some physiological adaptation regarding the use of

resources or investment in protection mechanisms against local stresses (Orchard *et al.*, 2010). Young individuals could, for example, adopt a more conservative growth strategy than those native to forest habitats due to the low supply of abiotic resources or the influence of biotic factors, such as herbivores. In studies in the Amazon Forest using species of the genus *Protium* sp., Fine *et al.* (2004) found significant differences in growth and mortality between species that occupy white sand or clay soil habitats. In the reciprocal transplant experiment, each species performed better in its habitat than in the opposite habitat, whether investing in defenses against herbivory and adopting conservative growth or investing in rapid growth in a more competitive environment and greater supply of resources (Fine *et al*, 2006).

In summary, while preliminary, our study presents significant results consistent with ecological speciation occurring in *restinga* environments. However, future studies will benefit from molecular analyses to test for genetic differences between populations and identify genes involved in reproductive isolation mechanisms. Our results also provide potentially helpful information for improving procedures for collecting seeds and transplanting seedlings for restoring degraded areas in *restinga* habitats. Depending on the origin of seeds and seedlings, their use may not be appropriate for plant restoration if the collection and destination environments are from mismatched habitats. In addition, planting seedlings in the wrong habitats can interfere with the local adaptation of species and ecological speciation.

We studied the initial stages of ecological speciation in *restinga* ecosystems for the first time in *Protium icicariba*. The fantastic environmental heterogeneity of *restinga* ecosystems represents an important driver of diversity, and our study contributes considerably to our understanding of the mechanisms of evolution in tropical plants. We conclude that environmental differences influence the flowering and fruiting period of the ecotypes, culminating in a partial asynchrony between the reproductive activity peaks of each population. Immigrant seeds demonstrate inferior germination performance in each habitat compared to native seeds. The growth and survival of seedlings were significantly better in the home habitat than in the contrasting habitat. These results are consistent with the hypothesis that habitat-mediated performance and survival reinforce local adaptation for habitat-specialist morphotypes. Given these results, we suggest the existence of basic conditions for the development of ecological speciation among populations of *P. icicariba* that occupy different *restinga* habitats.

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Authors' Contributions

Conceptualization, FCV and PCC; Data curation, FCV and RNSJ; Formal analysis and investigation, FCV; Supervision and visualization, PCC and BPBM; Writing - original draft, FCV and PCC; Writing - review and editing, FCV, RNSJ, BPBM, and PCC.

Conflicts of Interest

We declare that there are no known personal, scientific, commercial, political, or financial conflicts of interest that could have influenced the work reported in this manuscript.

Supplementary Material

The following online material is available for this article:

Table S1. Chemical and physical analysis of the soil in two habitats, open restinga and flooded forest of Paulo Cesar Vinha State Park, in municipality of Guarapari, state of Espírito Santo, Brazil.

Figure S1. Temperature and precipitation in Vila Velha municipality, state of Espírito Santo, Brazil (July 2018 to June 2021; INMET, 2022).

Figure S2. Method of germination in a common garden. In each plant habitat, four germination sites are established: 1, 2, 3 and 4. Two envelopes are buried in each site: one containing 30 seeds of native individuals of *P. icicariba* and an envelope containing seeds of immigrant individuals. SS = envelope with shrub seeds; FS = envelope with forest seeds.

Figure S3. Experimental method of transplanting seedlings. For each open restinga or flooded forest habitat, four planting sites are created: 1, 2, 3 and 4. In each site, 5 shrub seedlings (SS) and 5 forest seedlings (FS) of *P. icicariba* were replanted. The seedlings were oriented in a circular fashion and in pairs.

Figure S4. (A), (C) and (E): Individual of *P. icicariba* shrub. (B), (D) and (F): Individual of *P. icicariba* forest. (A) and (B) – Cross sections of the interveinal area of epidermal impressions. (C) and (D) – Cross sections of leaves. (E) and (F) – Stomatal complex of branches. Stomatal complex = cp, Cuticle = ct; Vessel element = v; Epidermis = ep; Fibers = fi; Palisade parenchyma = pp; Spongy parenchyma = pe; Uniseriate ray = ru. Scale: (A) and (B) = 10 μm; (C) and (F) = 40 μm.

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