RESEARCH ARTICLE

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Bicolored display of *Miconia albicans* fruits: Evaluating visual and physiological functions of fruit colors[1]

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PREMISE OF THE STUDY: Most bird-dispersed fruits are green when unripe and become colored and conspicuous when ripe, signaling that fruits are ready to be consumed and dispersed. The color pattern for fruits of *Miconia albicans* (Melastomataceae), however, is the opposite, with reddish unripe and green ripe fruits. We (1) verified the maintenance over time of its bicolored display, (2) tested the communicative function of unripe fruits, (3) tested the photoprotective role of anthocyanins in unripe fruits, and (4) verified whether green ripe fruits can assimilate carbon.

METHODS: Using a paired experiment, we tested whether detection of ripe fruits was higher on infructescences with unripe and ripe fruits compared with infructescences with only ripe fruits. We also measured and compared gas exchange, chlorophyll *a* fluorescence, and heat dissipation of covered (to prevent anthocyanin synthesis) and uncovered ripe and unripe fruits.

KEY RESULTS: Although the bicolored display was maintained over time, unripe fruits had no influence on bird detection and removal of ripe fruits. Ripe and unripe fruits did not assimilate CO_2, but they respired instead.

CONCLUSIONS: Since the communicative function of unripe fruits was not confirmed, seed dispersers are unlikely to select the display with bicolored fruits. Because of the absence of photosynthetic activity in ripe and unripe fruits and enhanced photoprotective mechanisms in ripe fruits rather than in unripe fruits, we could not confirm the photoprotective role of anthocyanins in unripe fruits. As an alternative hypothesis, we suggest that the bicolored fruit display could be an adaptation to diversify seed dispersal vectors instead of restricting dispersal to birds and that anthocyanins in unripe fruits may have a defense role against pathogens.

KEY WORDS anthocyanin photoprotection; frugivory; fruit contrast; fruit phenology; fruit photosynthesis; Melastomataceae; *Miconia albicans*

Fruit colors are traditionally viewed as an adaptation to increase detection of fruits by animals that disperse seeds (Kerner, 1895; Schmidt et al., 2004). Therefore, communication is the most commonly studied role of fruit colors because they facilitate detection and removal of ripe fruits by diurnal seed dispersers (Willson and Whelan, 1990; Schaefer and Schaefer, 2007; Cazetta et al., 2009; Valido et al., 2011). Diurnal frugivores use fruit color as a selection criteria, selecting, either more conspicuous red and black fruits against the leaf background (Schaefer and Schaefer, 2007; Cazetta et al., 2009; Melo et al., 2011) or those that reliably signal nutrients or antioxidants as a reward (Schmidt et al., 2004; Schaefer et al., 2008a; Schaefer and Ruxton, 2011).

Fleshy-fruited plants generally produce green unripe fruits that are not conspicuous against the green leaf background (Knight and Siegfried, 1983; Schaefer and Ruxton, 2011). As ripening progresses, green, unripe fruits change color as chlorophyll degrades, and eventually exhibit the colors of carotenoids and anthocyanins, which result in nongreen-colored, ripe fruits (Schaefer and Ruxton, 2011). Yet, some species produce fruits that are green when ripe.

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Typically, these fruits are large, inconspicuous, and dispersed by large mammals or bats (Knight and Siegfried, 1983; Cipollini and Levey, 1991). A green color seems disadvantageous for the attraction of visually guided frugivores, and yet these fruits retain chlorophyll and may contribute to the carbon requirements needed for plant reproduction, thereby reducing the costs of ripening (Cipollini and Levey, 1991; Aschan and Pfanz, 2003). For example, chlorophyll in fruits contributes to the production costs of developing peaches by fixing 15% of their carbon requirements (Pavel and DeJong, 1993).

A long-standing hypothesis predicts that species with relatively inconspicuous ripe fruits can improve the conspicuousness of fruit displays through nongreen secondary structures, such as bracts, capsules and stems (Schaefer and Schaefer, 2007; Schaefer et al., 2007). Likewise, colored unripe fruits occurring concomitantly with ripe fruits can increase detection and fruit removal by diurnal frugivores because of the temporally maintained bicolored fruit display (Stiles, 1982; Willson and Thompson, 1982; Fuentes, 1995; Cramer et al., 2003).

Anthocyanins contribute to blue and red to purple-black fruit colors (Steyn et al., 2002; Jaakola, 2013). This group of pigments has various functions, but few of them have been investigated in reproductive structures. In leaves, anthocyanins can play a role in photoprotection against excessive sunlight and ultraviolet radiation (Li et al., 1993; Gould, 2004). In the peels of apples and pears (Steyn et al., 2009) and in the red stems of *Cornus stolonifera* (Gould et al., 2010), anthocyanins prevent a decrease in photochemical performance under stress conditions such as low temperatures and excessive sunlight. This protective role is important for maintaining the photosynthetic capacity in leaves (Gould, 2004) and in other organs such as fruits, pedicels, and bracts (Cooney et al., 2015). However, except for cultivated species, studies focusing on fruit color rarely consider the alternative hypothesis of a photosynthetic role of pigments on fruit growth, rather than just the visual function of the colors (Cipollini and Levey, 1991; Aschan and Pfanz, 2003).

Unlike the typical fruit color pattern of bird-dispersed species, reddish unripe fruits and green ripe fruits are produced by *Miconia albicans* (Melastomataceae), a small tree from the Brazilian savanna (cerrado). These fruits are produced by obligatory apomixy, like many Melastomataceae species (Goldenberg and Shepherd, 1998), and seed dispersal is the only mechanism for gene flow (Goldenberg and Shepherd, 1998; Sales et al., 2013). Here we investigate the ecological function of this unusual bicolored fruit display, verifying its influence on fruit removal by seed dispersers and possible physiological functions of anthocyanin present in unripe fruits. To accomplish this goal, we (1) described the ripening pattern of *M. albicans* to verify the maintenance of the bicolored display over time, (2) tested the communicative function of red unripe fruits, (3) tested the photoprotective role of anthocyanins in reddish unripe fruits, and (4) verified whether green ripe fruits are photosynthetically active (carbon assimilation). We modeled the contrasts of ripe and unripe fruits and leaves and tested whether consumption of ripe fruits was higher in infructescences with both unripe and ripe fruits compared with infructescences with only ripe fruits. To prevent anthocyanin synthesis in unripe fruits, we covered unripe fruits with aluminum foil at the initial phase of fruit development and compared gas exchange and chlorophyll *a* fluorescence of covered and uncovered unripe fruits and of covered and uncovered ripe fruits.

If unripe fruits function as a communicative signal to seed dispersers we expected that birds would detect and remove more fruits from infructescences with unripe and ripe fruits than from those bearing only ripe fruits. Conversely, if anthocyanins have a photoprotective function in unripe fruits, we expected increased heat dissipation and lower light fraction used by the photosynthetic apparatus in unripe fruits with anthocyanins than in unripe fruits in which anthocyanin synthesis was prevented. Finally, we expected positive carbon assimilation in green, ripe fruits, or, at least, an increase in CO_2 assimilation rates and light fraction used by the photosynthetic apparatus in green, ripe fruits compared with reddish, unripe fruits.

MATERIALS AND METHODS

***Study site*—**We did this study in a woody cerrado with an area of 260 ha and altitude of 770 m a.s.l. in the municipality of Itirapina, São Paulo State, Brazil (22°10′31,41″S; 47°52′26,13″W). The vegetation is classified as a cerrado sensu stricto according to Coutinho (1978), a savanna with discontinuous woody strata reaching 6 to 7 m high or up to 12 m high in denser patches and a continuous herbaceous layer (Reys et al., 2013). The climate corresponds to the Cwa type according to the Köppen system (Köppen, 1948), with a dry winter from April to September and a wet summer from October to March. Average climatic data from 1972 to 2002 shows an annual rainfall of 1524 mm and mean annual temperature of 20.7°C (Camargo et al., 2011a).

Study species*—Miconia albicans* (Sw.) Triana (Melastomataceae) is a small tree up to 3 m high, distributed from southeastern Brazil to southern Mexico, and it is abundant in different physiognomies of the Brazilian cerrado and recently disturbed areas (Goldenberg, 2004; Allenspach and Dias, 2012). Flowers have a white-greenish cream color and a light smell, and are organized in inflorescences. They flower at the end of the dry season, peaking in September (M. G. G, Camargo and L. P. C. Morellato, personal observations based on 60 individuals observed monthly for 6 yr). They have poricidal anthers, thus requiring buzz pollination to release pollen grains (Buchmann, 1983; Renner, 1989; Goldenberg, 2004). In this species, pollen grains and floral visitors are rarely observed; fruits are produced by obligatory apomixy (Renner, 1989; Goldenberg and Shepherd, 1998; M/ G. G. Camargo, personal observations).

Fruits are small berries, 7.5 mm wide and 5.9 mm long, with an average of 34 seeds per fruit; seeds are 0.72 mm wide and 1.02 mm long (MGG Camargo, UNESP, personal observations). The pulp of ripe fruits is mainly composed of water (76.6%), sugars (13.4%) and proteins (2.17%) (Maruyama et al., 2007). Fruits are organized in infructescences. On the basis of fruit size and color, three stages could be distinguished during fruit development: (1) inconspicuous, greenish-gray, initially unripe fruits; (2) reddish, unripe fruits at an intermediate stage; (3) and fully expanded, green, ripe fruits. The intermediate unripe (hereafter "unripe") and ripe fruits have a persistent red calyx (Appendix S1a and b, see Supplemental Data with the online version of this article). *Miconia albicans* is one of the most common species at the study site (Reys et al., 2013) and is responsible for 30% of the annual production of animal-dispersed fruits in the local woody community (M. G. G. Camargo, unpublished manuscript). Ripe fruits are available mainly at the beginning of the wet season, peaking in November (based on unpublished

data from 6 yr of monthly phenological observations of 60 individuals). They are an important food source consumed by different bird species, mainly generalists (Allenspach and Dias, 2012), marsupials *Gracilinanus* spp. (Pereira et al., 2009; Camargo et al., 2011b), and fallen fruits are also collected by ants (Christianini et al., 2012). As far as we know, there is no information in the literature whether reddish, unripe fruits are consumed by birds.

***Frugivory*—**To access the importance of *M. albicans* fruits as a resource for birds and to see whether birds consume reddish, unripe fruits, we performed focal observations in 14 plant individuals. We observed bird activity from 05:00 to 18:00 during 5 d between 15 and 30 December 2010, during the peak of fruit production.

***Fruiting pattern*—**From September 2010 to March 2011, we quantified weekly the number of initial unripe, unripe, and ripe fruits on 30 individuals of *M. albicans* selected along a trail and at least 5 m from each other. To quantify the phenophases, we counted the number of fruits present on three infructescences in each individual at different stages and multiplied the mean number by the total number of infructescences present on each individual.

To estimate the life span of unripe and ripe fruits, we selected two infructescences on each of the 10 marked individuals that exhibited initial unripe fruits and marked 20 to 25 fruits on each one, totaling 445 marked fruits. We observed fruits every other day at the beginning and daily at the peak of the fruiting period.

***Color and contrast*—**Using a spectrometer (Ocean Optics USB 4000, Dunedin, Florida, USA), we measured the reflectance of the fruit skin of 30 unripe and 30 ripe fruits and from 30 leaves collected during the fruiting period. We recorded the light reflected within 300–850 nm, which includes the visual range for all frugivorous animals, including birds that are sensitive to UV light. With the mean reflectance spectrum of each structure, we calculated the chromatic and achromatic contrast between fruits and leaves and between ripe and unripe fruits. For that, we used visual models proposed by Vorobyev et al. (1998) for two kinds of avian visual systems according to their peaks of sensitivity for ultraviolet wavelengths: around 365 nm (UVS sensitive) or around 405 nm (VS sensitive). We performed these analyses using the R package Pavo (Maia et al., 2013).

***Fruit detection and removal*—**To analyze whether the presence of reddish, unripe fruits influences the removal of ripe fruits, we performed a paired experiment using two treatments in which each pair had a corresponding number of fruits: Treatment 1: infructescences with unripe and ripe fruits according to this proportion: 55% initial unripe fruits (i.e., inconspicuous), 25% unripe fruits, and 20% ripe fruits, according to the mean proportion of each fruit type in nonmanipulated plants (Appendix S1c). Treatment 2: infructescences with only ripe fruits (Appendix S1d), equivalent in number to the sum of unripe and ripe fruits present in the paired infructescences of treatment 1.

Considering a possible neighborhood effect, i.e., an influence of the density of fruiting plants in the vicinity and an influence of fruit crop size of individual trees on the detection of fruits by frugivores (Carlo and Morales, 2008; Christianini and Oliveira, 2009), we isolated the infructescences by removing and transferring them to a perch. Each perch was fixed on the top of a 2 m high wooden stake with a collector on the bottom (Fig. 1B). We repeatedly used 100 perches distributed along a trail, spaced 30 m apart. On the first day, we distributed the infructescences on the perches, counted the number of ripe fruits in each treatment, and replaced the dry and damaged fruits or infructescences. An infructescence was regarded as being detected if at least one ripe fruit was removed. If a perch was detected, it was used again only after 4 d, to avoid subsequent visits based on memory rather than fruit detection. We conducted the experiment between December 2012 and January 2013, during the fruiting peak of *M. albicans*, totaling 30 d that correspond to four to five continuous days of data collection with 1 d between each data collection. In the first day of each data collection, we randomly selected perches and the corresponding treatment using from 40 to 60 perches. All 100 perches were used during the experiment, and 77 were detected at least once.

For each perch, we estimated canopy openness, number of *M. albicans* reproductive individuals present in a radius of 5–10 m from the perch, and their respective tree circumference 3 cm from the soil. These data allowed us to verify the influence that the environment and the presence of fruiting *M. albicans* individuals around the perch could have on fruit removal. To estimate canopy openness, we took photos using a hemispherical lens "fisheye converter" (Nikon FC-E9) coupled to a digital camera (Nikon Coolpix, model 8700) that was positioned above each perch. We transferred these photos to the Gap Light Analyzer 2.0 software (Frazer et al., 1999), which provides data on the percentage of canopy openness according to the proportion of black or white pixels in the hemispherical photo.

***Photosynthesis and anthocyanins*—**To verify the photoprotective role of anthocyanins, we first covered some unripe fruits with aluminum foil to prevent anthocyanin synthesis and left some uncovered. After that, we compared CO_2 assimilation rate (A), chlorophyll *a* fluorescence, and heat dissipation (1) between unripe fruits with anthocyanins (uncovered) and unripe fruits without anthocyanins (covered) and, after fruit development, (2) between covered and uncovered ripe fruits.

Considering that the synthesis of anthocyanins usually requires exposure to light (Mancinelli, 1985; Steyn et al., 2009; Jaakola, 2013, Cooney et al., 2015), we selected 60 infructescences (on 30 plant individuals) and covered 8 to 10 initially unripe fruits with aluminum foil to block sunlight. We left an aperture on the underside of the aluminum foil to allow fruit gas exchange and prevent fruit abortion. The remaining fruits were completely exposed (uncovered) to sunlight (Appendix S1e). After fruit development, which was assessed based on fruit size, we measured the reflectance spectra of 20 covered and uncovered unripe fruits using a spectrometer (Ocean Optics USB4000). Because anthocyanins are responsible for the reddish color of unripe fruits, we calculated the ratio of red to green fruits for covered and uncovered unripe fruits and used this ratio as an estimation of fruit anthocyanin content (Gamon and Surfus, 1999; Schaefer and Braun, 2009; Gould et al., 2010).

To verify any photosynthetic activity in ripe and unripe fruits, we evaluated fruit carbon assimilation by measuring the CO_2 assimilation rate (A) in response to an artificial light inside the chamber that provided photosynthetic photon flux density (PPFD) similar to the external environment and also in response to increasing the artificial PPFD (photosynthetic light curves). For this, we measured A of seven to nine covered and uncovered ripe and

unripe fruits on 3 days during the ripening period. Measurements were performed in the field, using a portable open gas exchange system (LI-6400xt; LI-COR, Lincoln, Nebraska, USA) coupled to a fluorometer chamber (LCF 6400-40; LI-COR) that was also used for measuring chlorophyll *a* fluorescence.

For gas exchange measurements, in vivo fruits were accommodated within the chamber, and measurements were performed between 09:00 and 12:00, as this interval seems to exhibit mild temperatures and best PPFD for gas exchange (Feistler and Habermann, 2012). The measurements were taken between 10 December 2013 and 06 January 2014, with ambient temperature around 23°C (±1.6) on the days of data collection. The PPFD within the chamber was provided with an artificial red (90%) blue (10%) LED light source (6400-02B, LI-COR; Tennessen et al., 1994; Shimazaki et al., 2007) held on the top of the chamber. The PPFD was set according to the PPFD observed in the external ambient (≈ 600 μmol photons·m^{-2}·s^{-1}), the CO_2 concentration entering in the leaf chamber was set to 390 μmol·mol^{-1}, and the chamber flow was maintained around 450 μmol·s^{-1}. The mean temperature in the chamber was 30°C (± 2.15). Light curves (*A*/PPFD) were obtained by varying the PPFD from 1200 μmol·m^{-2}·s^{-1} to zero (Habermann et al., 2011). After measurements, we estimated the area (cm^2) of fruits that comprised the sample within the chamber and recalculated *A* based on the corrected fruit surface. For this, the diameter of every fruit was registered, and the half sphere surface was calculated because only the upper half of the fruits were exposed to the light coming from the top of the chamber. The sum of the half sphere surfaces was used as the corrected area logged in the LI-6400xt data analysis program.

To estimate the photoprotective role of anthocyanins, we measured chlorophyll *a* fluorescence and calculated the fraction of absorbed light used in photochemical activity [$P = (Fm' - Fs)/Fm'$], which is equivalent to the effective quantum yield of photosystem II (ΦPSII), the heat dissipation in the light-gathering pigment antenna, which collects light and transfers the energy to the reaction center complex ($D = 1 - Fv'/Fm'$) and the heat dissipation in reaction centers of photosystem II, PSII [$E = (1 - qP)(Fv'/Fm')$] according to da Veiga and Habermann (2013). We used saturating light pulses of 7000 μmol photons·m^{-2}·s^{-1} for 0.7 s. For these equations, Fv′ is the variable fluorescence between the maximal (Fm′) and minimal (Fo′) fluorescence of light-adapted fruits, and Fs is the steady-state fluorescence of light-adapted fruits. Photochemical quenching (qP) was calculated as [(Fm′ − Fs)/(Fm′ − Fo′)], as adapted from Maxwell and Johnson (2000). Chlorophyll fluorescence is the re-emitted and nonutilized light in photosynthesis, and it is widely used as a method to estimate the performance of PSII under abiotic stresses (Maxwell and Johnson, 2000). Heat dissipation in the light-gathering antenna (*D*) and reaction centers of PSII (*E*) and the fractions of absorbed light used in photochemical activity (*P*) provide useful information about photoprotection (increased *D* and *E*) or efficiency of light reactions in photosynthesis (high *P*) (Maxwell and Johnson, 2000).

FIGURE 1 (A) *Miconia albicans* infructescence with few initial unripe fruits (indicated by an arrow), reddish, intermediate unripe fruits and green, ripe fruits. Perch used for detection and fruit removal experiments (B).

***Data analyses*—**To assess the influence of the presence of unripe fruits on the number of detected infructescences and fruits removed, we used generalized linear models (GLM), specifically, binary logistic regressions for infructescences detection and Poisson GLM for the number of fruits removed. We entered the number of ripe fruits at each perch as a covariate to assess whether the variation in the number of ripe fruits influenced the likelihood that an infructescence was detected. We also used Poisson GLM to verify the influence of the canopy openness above the perches and the number and size of reproductive *M. albicans* individuals around the perches on the detection rate of infructescences and in the number of ripe fruits removed in each perch. All GLM models used are presented in the Appendix S2 (Appendix S2, see online Supplemental Data).

To detect possible differences in fruit color indicating the synthesis of anthocyanins, we compared covered and uncovered unripe fruits reflectance spectra between 300 to 670 nm and the reflectance spectra in the red band (between 600 to 670 nm) using Wilcoxon tests (T). We used *t* tests to compare red: green ratio and CO_2 assimilation rate, fractions of absorbed light used in photochemistry (P), heat dissipation in the antenna (D) and in reactions centers (E) of covered and uncovered unripe fruits (Zar, 1999).

We performed all analyses in the program R, version 3.1.2 (R Development Core Team, 2009).

RESULTS

***Fruiting pattern*—**The 30 observed individuals produced fruits from 22 October 2010 to 30 March 2011 (Fig. 2). The number of initially unripe fruits peaked on 12 November 2010 and were observed until 17 February 2011, with a mean of 7209 fruits per individual. Unripe and ripe fruit production overlapped, peaking on 30 December 2010, but they could be observed from 3 December 2010 to 30 March 2011, with a mean of 500 unripe and 447 ripe fruits per plant.

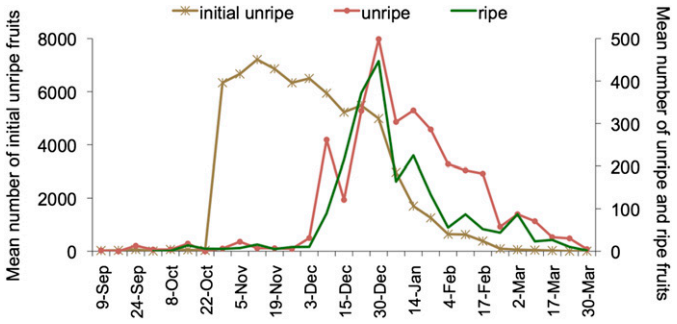

FIGURE 2 Fruiting patterns of 30 *Miconia albicans* individuals observed between September 2010 and March 2011 in wood cerrado–savanna vegetation in southeastern Brazil.

From 445 initially unripe fruits that were marked, 382 (85.8%) turned into unripe fruits, and 346 (77.7%) ripened and were monitored until the abscission of ripe fruits. Unripe fruits lasted for 7 ± 5.9 (SD) days, and ripe fruits for 4 ± 2 (SD).

***Infructescence detection and fruit removal*—**During the 219 h of focal observation, we registered 194 visits of 26 bird species removing ripe fruits of *M. albicans* (online Appendix S3). Unripe fruits were not consumed or removed.

Reflectance data confirmed the difference in unripe and ripe fruit color previously observed in the field (Fig. 3). There was almost no overlapping in the reflectance data of leaves and fruits with the highest chromatic and achromatic contrasts between unripe fruits and leaves and not between ripe and unripe fruits (Fig. 3 and Table 1).

Treatments did not influence the number of infructescences detected ($z = 1.28$, $p = 0.20$). Perches of treatment 1 (infructescences with ripe and unripe fruits) were detected 72 times, and 145 fruits were removed, while perches of treatment 2 (only ripe fruits) were detected 88 times, and 310 fruits were removed. Yet, treatments did influence the number of fruits removed ($z = 2.9$, $p < 0.01$), which was higher in treatment 2 with only ripe fruits. We found no influence of the canopy openness or the number and circumference size of *M. albicans* reproductive individuals around the perches on the detection of infructescences ($z = 0.59$, $p = 0.55$) or on fruit removal ($z = 0.51$, $p = 0.60$).

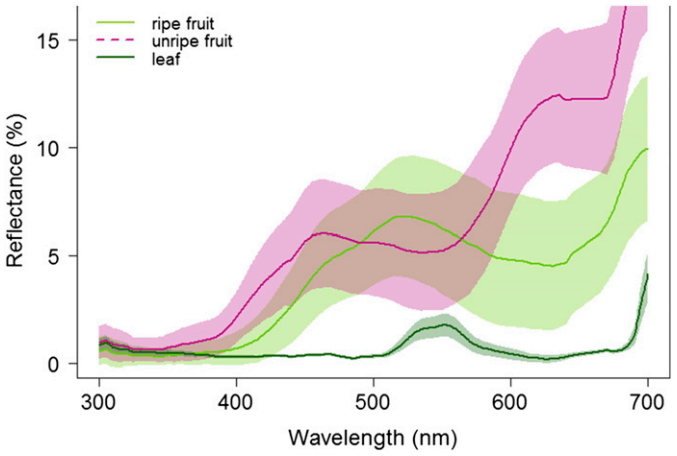

FIGURE 3 Mean reflectance spectra (lines) and standard deviation (shading) of *Miconia albicans* ripe fruits, unripe fruits, and leaves.

TABLE 1. Chromatic and achromatic contrast between ripe fruits, leaves, unripe fruits, and fruit persistent calyx of *Miconia albicans* according to a bird's visual system model considering sensitivities for ultraviolet light.

	Chromatic UVS		Chromatic VS		Achromatic	
Stage	Ripe fruit	Leaf	Ripe fruit	Leaf	Ripe fruit	Leaf
Ripe fruit	—	23.1	—	14.1	—	36.9
Unripe fruit	11.41	26.3	11.81	20.8	5.63	42.5

Notes: UVS = visual bird system with peak sensitivity for ultraviolet wavelengths around 365 nm, VS = visual bird system with peak sensitivity for ultraviolet wavelengths around 405 nm.

***Photosynthesis and anthocyanins*—**Covered and uncovered unripe fruits did not differ in the red to green fruits ratio (t test, $t = 0.02$, df = 35.4, $p = 0.98$).

The CO_2 assimilation rate was negative in covered and uncovered unripe and ripe fruits, indicating that respiration and no photosynthetic activity (Fig. 4). Respiration was higher in covered compared to uncovered unripe fruits (t test, $t = 2.47$, df = 39.6, $p = 0.02$); for ripe fruits, respiration differed marginally between covered and uncovered fruits (t test, $t = 2.00$, df = 38.70, $p = 0.05$). Respiration (negative CO_2 assimilation) did not respond to the variation in PPFD (A/PPFD curves) either in unripe (Fig. 5A) or ripe fruits (Fig. 5B). Although we did not check the presence of stomata on fruits, Cortez and Carmello-Guerreiro (2008) described stomata in the pericarp of *Miconia albicans* fruits. We found positive stomatal conductance for unripe (0.06 ± 0.06 mol·m^{-2}·s^{-1}) and ripe fruits (0.07 ± 0.5 mol·m^{-2}·s^{-1}). Therefore, we must consider that these responses could be related to gas exchange occurring on the fruit's epidermis.

Fluorescence variables (P, D, and E) were significantly different between covered and uncovered fruits, regardless of the ripening stage of the fruit (Fig. 6). Relative to covered fruits, uncovered ones had higher fractions of absorbed light used in photochemistry, P (t test, $t = 5.66$, df = 41.93, $p < 0.01$ for unripe and $t = 3.58$, df = 45.97,

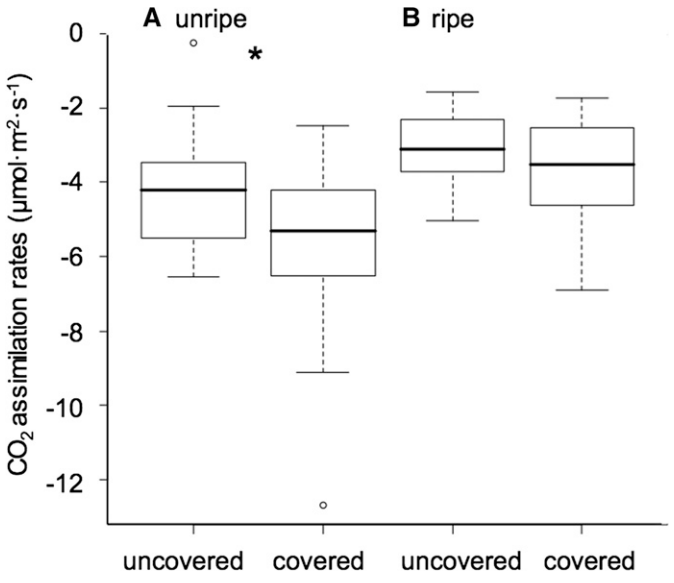

FIGURE 4 Box plot of CO_2 assimilation rates in uncovered and covered unripe and ripe fruits of *Miconia albicans*. * Significantly different according to t test.

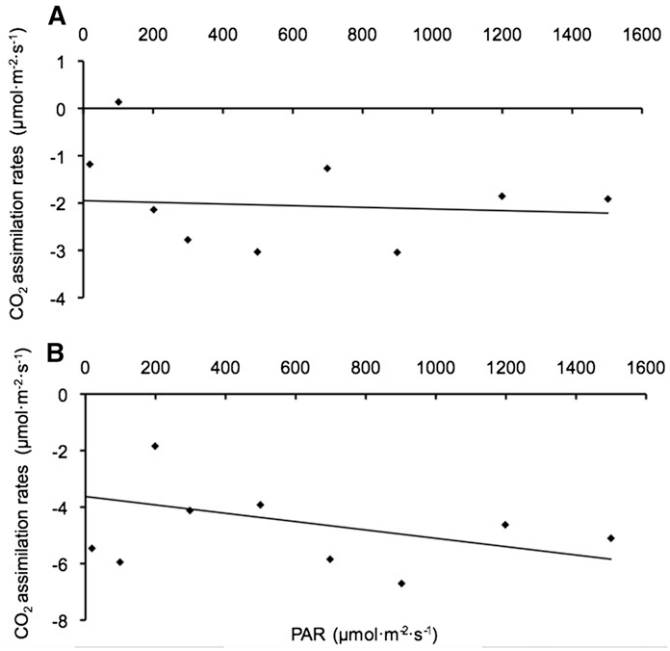

FIGURE 5 Light response curve representing CO_2 assimilation rates for different photosynthetic photon flux densities (PPFD) of (A) unripe and (B) ripe fruits of *Miconia albicans*.

$p < 0.01$ for ripe fruits; Fig. 6A), and higher heat dissipation in the antenna, D (t test, $t = 4.68$, df = 38.32, $p < 0.01$ for unripe and $t = 2.71$, df = 43.86, $p = 0.01$ for ripe fruits; Fig. 6B). The opposite (covered > uncovered) occurred for dissipation in reactions centers, E (t test, $t = -6.24$, df = 39.74, $p < 0.01$ for unripe and $t = -3.53$, df = 41.91, $p < 0.01$ for ripe fruits; Fig. 6C).

DISCUSSION

We observed the presence of reddish, unripe fruits throughout the fruit season, confirming the persistence of the bicolored of *M. albicans* infructescences. However, our results did not support a communicative function of the unripe fruits, because they did not increase ripe fruit contrast, detection, or removal. Similarly, a photoprotective role of anthocyanins in fruits could not be confirmed due to the enhanced photoprotective mechanism observed in green, ripe fruits rather than in reddish, unripe fruits, with increased heat dissipation in the light-gathering antenna (D) in ripe fruits. Though not confirmed here, our study highlights the importance of considering alternative functions of fruit color beyond plant–animal communication and the role of anthocyanins in nonvegetative plant organs, factors that influence fruit color evolution (Schaefer and Schaefer, 2007).

Our results showed that different bird species consume *M. albicans*, mainly generalists, as also observed by Allenspach and Dias (2012), and the birds we observed fed exclusively on ripe fruits, differentiating them from the reddish, unripe ones. In general, birds have an innate preference for red fruits, which are more common, conspicuous, and preferentially selected over other colors, such as white and green (Chen et al., 2004; Schaefer et al., 2008b; Schaefer and Ruxton, 2011). However, fruit color preferences are inconsistent in birds because color-reward associations

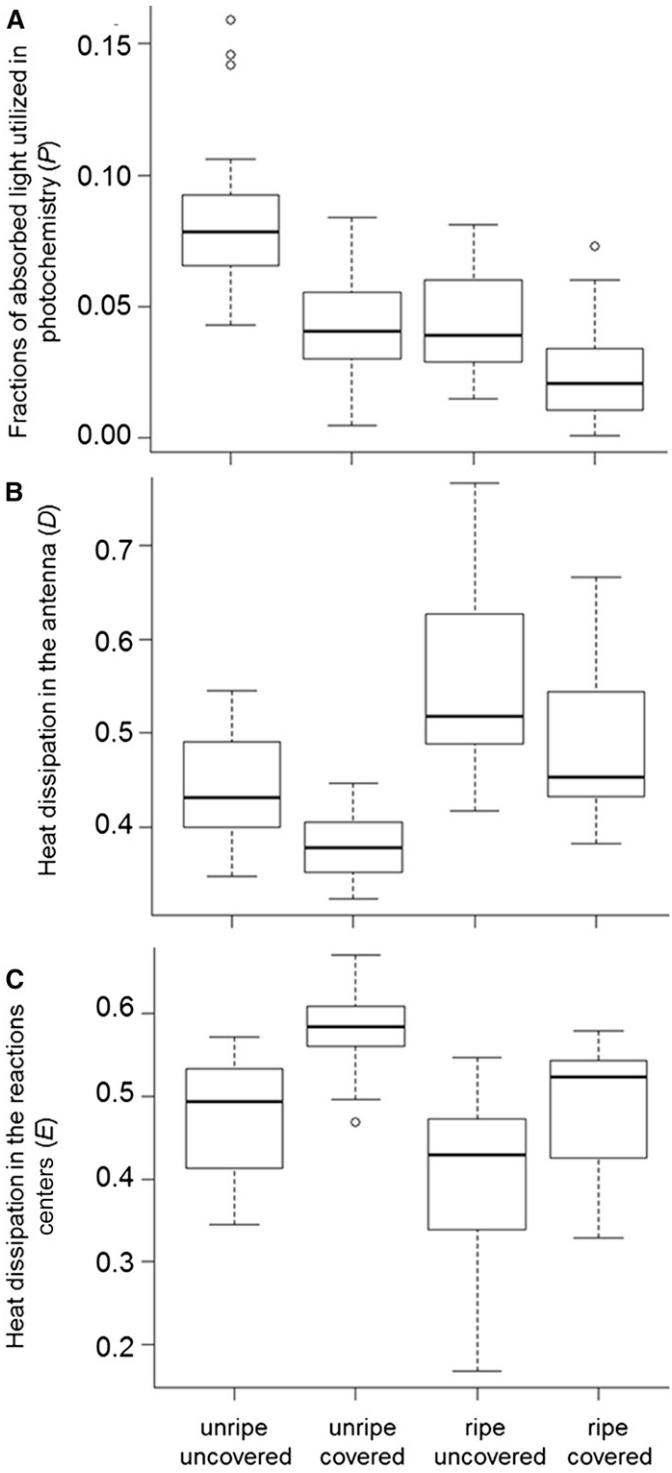

FIGURE 6 Chlorophyll *a* fluorescence variables, (A) absorbed light used in photochemistry, (B) heat dissipation in the antenna, and (C) heat dissipation in reactions centers in uncovered and covered unripe and ripe fruits of *Miconia albicans*. Values for each variable differed significantly between covered and uncovered fruits according to a *t* test.

can be spatially variable, and learning plays an important role in fruit selection (Schaefer et al., 2008b; Schaefer and Ruxton, 2011). The removal of only green, ripe fruits of *M. albicans* could

be related to associative learning by birds, based on a larger size or green color of fruit.

Although unripe fruits do not directly enhance the contrast of ripe fruits, unripe fruits have a higher contrast than the ripe ones against leaves, increasing the overall contrasts of the fruit display. However, this bicolored display of *M. albicans* infructescences seems to have no influence on fruit detection and removal. Since unripe, reddish fruits did not influence the probability that infructescences were detected by birds, the higher fruit removal in infructescences with only ripe fruits is probably related to the higher number of ripe fruits available in this treatment. Instead of serving as a communicative function, the bicolored display of *M. albicans* could be related to limited resources for synchronous fruit maturation or to a sequential anthesis strategy in the inflorescence, conferring asynchronous fruit ripening in the infructescence (see also Amsberry and Steffen, 2008).

In general, there is no consensus on the effects of bicolored display of ripe and unripe fruits on fruit detection and removal by birds. For example, higher fruit removal from bicolored displays of black, ripe and red, unripe fruits was reported for *Prunus serotina* and *Phytolacca americana (*Willson and Melampy, 1983) and also a higher removal rate for blue *Psychotria pittieri* ripe fruits when associated with an artificial red bract (Wenny, 2003). Similar results are reported for infructescences with only red, unripe fruits associated with infructescences with only green, ripe fruits of *Pistacia terebinthus* (Fuentes, 1995). In contrast, fruit removal was higher in infructescences with only green, ripe fruits of *Pistacia terebinthus* compared with treatments that offered red, unripe and green, ripe fruits together in the same infructescence (Fuentes, 1995). Amsberry and Steffen (2008) found higher fruit removal by birds in infructescences with only red, ripe fruits than when combined with white and pink, unripe fruits of *Ardisia nigropunctata*. Finally, Burns, (2005) also found no preference for experimental bicolored display in *Rubus spectabilis*.

Some of the contrasting results in the selection of bicolored fruit displays could be related to differences in the methods used. First, all studies mentioned above based their hypotheses on fruit colors as they are perceived by humans and not by seed dispersers. Second, only the number of fruits removed was compared between treatments, without considering the detection of infructescences. Third, the experimental design used by these studies differs among them, with infructescences being paired or randomly distributed, and with variations in the number of fruits of different colors offered in each experimental infructescence (e.g., Willson and Melampy, 1983; Amsberry and Steffen, 2008). Finally, in most studies fruits that drop beneath the infructescences are often not differentiated from those actually removed by birds. One bias of our study could be the absence of protection against nonvisually guided frugivores, such as marsupials (*Gracilinanus* spp.), that consume *M. albicans* fruits (Pereira et al., 2009). However, during the experiment, we did not observe any trace of mammals feeding on experimental infructescences.

With regard to the photoprotective role of anthocyanins, our results did not show differences in anthocyanin content between covered and uncovered unripe fruits. Therefore, we concluded that sunlight might not be the most important factor inducing anthocyanin synthesis in *M. albicans*, as this biosynthesis is also controlled by genetic factors (Jaakola, 2013). Contrasting with our predictions, the change from reddish to green during ripening did not cause any increase in photosynthetic performances in ripe fruits. Neither ripe nor unripe fruits assimilated CO_2, but they respired instead. Thus, in *M. albicans*, chlorophyll in fruits did not contribute to the maintenance costs of these organs. Even as PPFD increased, the respiration rate did not decrease in *M. albicans* fruits, indicating that respiration is self-controlled rather than environmentally controlled in these fruits. This result contrasts with previous studies on larger fruits (Cipollini and Levey, 1991), which may be more costly to produce and maintain. As such, we rule out the hypothesis that the adaptive significance of the fruit color shift toward green, ripe fruits is increasing their photosynthetic abilities.

Uncovered fruits had enhanced light reactions in the photochemical "apparatus" because *P* and *D* were higher in relation to fruits that were protected from sunlight, indicating that electron transporters are more active (not oxidized; Baker, 2008) in the presence of light, suggesting that light does play a role in the functioning of the photochemical apparatus of *M. albicans* fruits. During the ripening of nongreen, ripe fruits, less chlorophyll fluorescence is generally expected as chlorophyll is degraded, with corresponding changes from green to yellow (Bron et al., 2004). For *M. albicans*, the opposite is expected as ripe fruits become green. However, in the current study, *P* consistently decreased and *D* increased as fruits ripened, suggesting that the photochemical activities to provide NADPH and ATP to the carboxylation requirement are attenuated (Baker, 2008). In addition, the efficiency of photosystem II decreased as fruits ripened, as evidenced by decreased values of *P* and increased values of *D* during ripening, reinforcing a low photochemical activity of green ripe fruits. This finding strengthens our conclusion that ripe fruits, although green, do not contribute to photosynthates for fruit development. Given that the observed photoprotective mechanism (increased *D*) is enhanced in ripe fruits, rather than in unripe fruits that are supposedly rich in anthocyanins, we could not confirm the photoprotective role of anthocyanins in reddish, unripe fruits. Although the unexpected higher *P* values in reddish, unripe fruits in relation to green, ripe fruits could indicate that some photochemical activity occurs in unripe fruits, this phenomenon can be a consequence of the anthocyanin presence in the unripe fruits, which sometimes can cause an overestimate of ΦPSII values (Pietrini and Massacci, 1998). In a similar experiment, Cooney et al. (2015) compared *Sambucus nigra* infructescences with green and red peduncles and did not confirm a communicative role of anthocyanins. However, contrary to our results in unripe fruits of *M. albicans*, anthocyanins exerted a photoprotective function, which probably is the driver for anthocyanin accumulation in the peduncles of *Sambucus nigra* (Cooney et al., 2015). Although various roles are frequently described for anthocyanins in nonreproductive plant organs, it is still necessary to verify possible different roles of anthocyanins in fruits to understand evolutionary drivers of fruit color.

Taking into account the importance of fruit removal and seed dispersal for gene flow in *M. albicans* due to the obligatory apomictic reproductive system, we suggest that the bicolored fruit display may be a strategy toward a diversification of seed disperser vectors, instead of restricting dispersal to birds, improving seed dispersal possibilities and efficiency. Our assumption is supported by the importance of *M. albicans* fruits for some cerrado marsupials that are potential seed dispersers of this species (Pereira et al., 2009; Camargo et al., 2011b). One example is the high frequency of *M. albicans* in fecal samples of *Gracilinanus agilis*, in which *M. albicans*, *M. ferruginata*, and *M. pohliana* were present in 44% of 186 samples (Camargo et al., 2011b).

A possible alternative role for anthocyanins on reddish unripe fruits of *M. albicans* might be related to fruit defense against pathogens and herbivores, as already reported for leaves (Gould, 2004; Cooney et al., 2012) and nonvegetative structures in plants. Irwin et al. (2003) demonstrated that anthocyanin color of *Raphanus sativus* flowers works as a cue to deter herbivores. Anthocyanins in combination with defensive compounds, such as as thiarubrine A, can also indicate the presence of defensive compounds in immature fruits (Gould, 2004) or promote crypsis in red fruits to frugivorous insects since most of them are not sensitive to red band wavelengths (Briscoe and Chittka, 2001). The alternative hypothesis of a defensive role for anthocyanins might be reinforced by field observations of the absence of herbivory of unripe fruits of *M. albicans* and the high ripening success, just 10% failure, even with the frequent presence of fungi and galls (Viana et al., 2013; Appendix S1f and g) in leaves, flower buds, and fruits.

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