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INSIGHTS

Red coloration in young tropical leaves associated with reduced fungal pathogen damage

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ABSTRACT

The adaptive significance of red coloration in tropical forest leaves remains unclear. *In vivo* assays show that there is a significant negative correlation between anthocyanin pigments in young leaves and fungal pathogen damage. This supports a previous hypothesis that anthocyanins may protect young leaves from fungal damage during the vulnerable period of leaf expansion.

Abstract in Spanish is available with online material.

Key words: anthocyanins; *Calonectria* sp; canopy crane system; Panama; tropical wet forest.

In the humid tropics, approximately one-third of woody plant species delay greening of their leaves via delayed production of chlorophyll pigments or chloroplast development (Coley & Kursar 1996), and often exhibit a dramatic red coloration in their young leaves (Coley & Barone 1996). This red coloration is attributed to high concentrations of anthocyanin pigments in mesophyll and epidermal cells produced during leaf expansion that disappear as the leaf matures (Coley & Barone 1996). Anthocyanin pigments have been well studied in plants for the role they play in attracting pollinators and frugivores (Lattanzio *et al.* 2006) but their functional significance in young developing leaves is not entirely understood.

Several hypotheses have been proposed to explain the adaptive significance of anthocyanin pigments in young leaves. Anthocyanins may protect leaves against photoinhibition (Gould *et al.* 1995, Close & McArthur 2002, Close & Beadle 2003) or act as a photoprotectant for plant organelles against UV radiation (Lee & Lowry 1980, Burger & Edwards 1996). These two hypotheses are unlikely in the tropics. Xanthophylls, and not anthocyanins, are known to protect against photoinhibition in tropical forests (Krause *et al.* 1995). In addition, if anthocyanins were photoprotective, we would expect to find red leaves more often in the canopy—where brighter conditions exist—relative to the understory, but this has not been the case (Dominy *et al.* 2002). Alternatively, it has been suggested that anthocyanins defend against herbivory, with their pigments providing cryptic coloration to insect herbivores that are blind to the red part of the light spectrum (Dominy *et al.* 2002, Karageorgou & Manetas 2006). Although anthocyanin pigments may act as a deterrent to herbivores, few studies have examined the role of anthocyanins in protecting plants against pathogens.

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In tropical rain forests, fungal pathogens play a major role in shaping and maintaining plant diversity and species composition (Bagchi *et al.* 2014), negatively affecting many plants through reduced host growth or reproduction (Gilbert 1995, Bagchi *et al.* 2014, García-Guzmán & Heil 2014). Plants are known to protect themselves from pathogens through the production of phenolic compounds such as flavonoids, which have anti-fungal properties (Alcerito *et al.* 2002, Lattanzio *et al.* 2006, Treutter 2006). Seedlings with anthocyanin pigments—a sub-group of flavonoids—have been associated with higher survival rates compared to seedlings with green leaves (Queenborough *et al.* 2013). This could be driven in part by anthocyanin mediated protection against pathogens. Coley and Aide (1989) found that leaf-cutter ants preferentially picked up leaves without anthocyanins. They further hypothesized that anthocyanins may protect against fungal pathogen attack during the vulnerable period of leaf expansion, but no follow-up study has been conducted. Our study explores this hypothesis by performing *in vivo* bioassays with a generalist fungal pathogen and young leaves varying in anthocyanin content. In a wet evergreen forest in Panama, we examine the question, do anthocyanins in developing young leaves protect against fungal pathogen damage?

The study was conducted in Parque Nacional San Lorenzo (PNSL) in the Republic of Panama. Parque Nacional San Lorenzo (9°17′N, 79°58′W) encompasses 5.96 ha of tropical, wet, evergreen forest on the Caribbean coast of Panama. This site receives approximately 2700–3000 mm of annual rainfall and has a mean annual temperature of 26°C with little variation among months (Condit *et al.* 2004). The site plot is 400 × 100 m with a 140 × 140 m contiguous area to the left side of the southernmost hectare. A canopy research crane managed by the Smithsonian Tropical Research Institute found in the center of this 140 × 140 m plot gives canopy access for an area of 0.9 ha (Condit *et al.* 2004).

To determine the best generalist pathogen to use in our field inoculations, we tested eight fungal pathogens and control plugs without fungal pathogens on three leaves from various tree species near our lab in Gamboa, Panama. A species of *Calonectria* was shown to infect all of the leaves from all trees sampled. Several species of *Calonectria* are known necrotrophic plant pathogens associated with a wide range of agricultural and forestry crops worldwide (Lombard *et al.* 2010), with leaf spotting being the most common disease symptom. Five tree species in PNSL were chosen for fungal inoculation trials: *Brosimum utile* (Moraceae), *Coccoloba* sp. (Polygonaceae), *Perebea angustifolia* (Moraceae), *Protium panamense* (Burseraceae), and *Manilkara bidentata* (Sapotaceae). These tree species were chosen because they were flushing an abundance of young leaves, showed variation in leaf color, and were easy to access using the canopy crane system.

We prepared pathogen inoculum following Gilbert and Webb (2007). This involved placing caps of 1.8 ml cryovials (Sigma, St Louis, Missouri, U.S.A.) inside large glass Petri dishes with the deep end of the cryovial cap facing upward. The cryovial caps were autoclaved for 20 min and, using a pipette, we transferred sterile, molten, 2% malt extract agar (MEA) into each cap until full. Once the agar had solidified, we placed a small piece (~2–3 mm^2) of colony cut from 1-wk old *Calonectria* sp. cultures grown on 2% MEA on solidified caps. Petri plates containing caps with fungi were placed inside an incubator (23°C) and allowed to grow for 5 d before inoculations. Once fungi had grown to cover the caps, we removed the caps and placed them in sterile, sealed Whirl-pak bags (Nasco, Fort Atkinson, Wisconsin, U.S.A.) for transport to the study site.

We randomly selected ten young leaves from five tree species ($N = 100$) from both the forest understory and canopy for inoculation trials. We surveyed trees for newly flushed leaves by visually inspecting for leaf color and size (30–50% of full size), relative to older, mature leaves. Before pathogen inoculation, we measured anthocyanin content on each leaf at three sites: apically, medially, and basally, and averaged measurements together. About 80–90 percent of anthocyanins found in leaves are composed of cyanidin, a type of anthocyanin with a transmittance wavelength of 520–530 nm (Coley & Aide 1989, Onslow 2014). We measured anthocyanin content using the ACM-200 plus (Opti-Sciences Inc., Hudson, New Hampshire, U.S.A.). The ACM-200 uses the ratio of % transmittance at a wavelength of 931 nm (infrared wavelength to compensate for sample thickness) and 525 nm (anthocyanin transmittance) to calculate an anthocyanin content index (AIC) value (AIC = % transmittance at 931 nm/% transmittance at 525 nm). We used sterile forceps to remove the inoculum cap from the Whirl-pak, pressed the cap against the underside of the leaf, and clipped it in place with a bent, snap-on hair clip (Scunci, East Windsor, New Jersey, U.S.A.). Leaves were harvested after 4 d and the area of visible necrosis damage was measured using the software Image J (NIH, U.S.A.). All infected leaves were removed from the forest and transported to our lab in Gamboa for measurement and disposal.

A Shapiro–Wilk's test showed that the continuous variables of leaf damage and anthocyanin content were not normally distributed. Transformation did not remedy their non-normality. A non-parametric Levene's test verified the equality of variances in the samples. Non-parametric Spearman's rank correlation was performed to examine the relationship between foliar fungal pathogen damage and anthocyanin content in leaves. In addition, a Mann–Whitney *U*-test was used to test for differences in anthocyanin content and pathogen damage between the forest canopy and understory. The data were analyzed in SPSS v. 21.1 for Windows (SPSS, Chicago, Illinois, U.S.A.).

Sixty-two leaves were recovered and all leaves had visible necrosis damage. The inoculation trials using the fungal pathogen *Calonectria* sp. showed a significant negative correlation between anthocyanin content in young leaves and necrosis damage by the fungal pathogen ($r = -0.459$, $N = 62$, $P < 0.001$, Fig. 1). The relationship appears as a negative exponential (Fig. 1), however, we did not fit a negative exponential line because our data violated the normality assumption. Overall, an increase in anthocyanin content led to a decrease in necrosis damage. Tree species varied in the amount of anthocyanin content and necrosis damage (Table 1). Among the tree species, *Protium panamense* had the highest anthocyanin content in both the forest canopy and understory but had middle range values for leaf damage (Table 1). Young leaves of *Coccoloba* sp. also had higher levels of anthocyanins and showed the lowest necrosis damage in the canopy (Table 1).

A comparison of young leaves showed that there was no significant difference in anthocyanin content between the forest understory (median = 3.26) and the forest canopy (median = 4.63), ($U = 359.0$, $P = 0.148$). However, when comparing leaf damage, young leaves in the canopy (median = 2.49) had

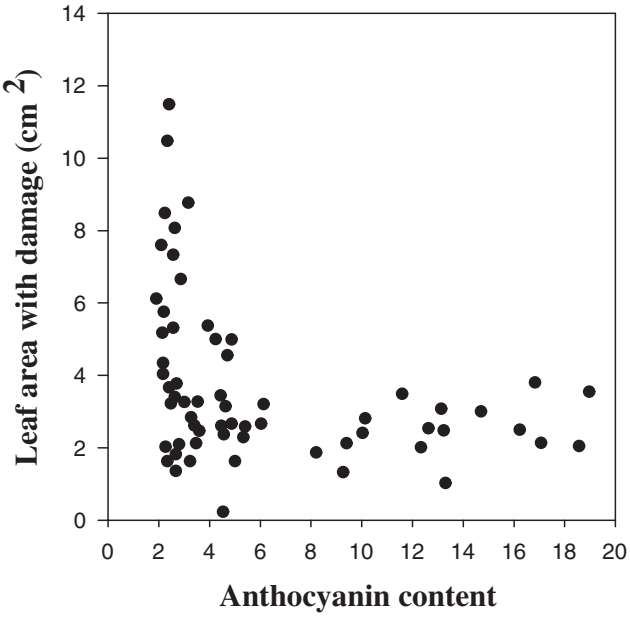

FIGURE 1. Relationship between anthocyanin content in young leaves and pathogen induced damage. Data are shown for 62 leaves of five tropical tree species from both the forest canopy and understory.

TABLE 1. *Data for mean (±SE) anthocyanin content (AIC) and fungal pathogen damage (cm^2, leaf area) for five tree species located in the forest canopy and understory at Parque Nacional San Lorenzo.*

	Canopy			Understory		
Species	*N*	AIC	Damage	*N*	AIC	Damage
Brosimum utile	9	4.10 (0.35)	2.62 (0.31)	5	4.32 (0.55)	2.82 (0.34)
Coccoloba sp.	5	9.85 (0.69)	1.95 (0.18)	5	4.60 (0.37)	3.13 (0.48)
Protium panamense	9	14.8 (1.03)	2.79 (0.18)	3	7.14 (2.75)	4.30 (0.89)
Manilkara bidentata	5	2.55 (0.07)	2.29 (0.43)	7	2.48 (0.68)	5.52 (0.94)
Perebea angustifolia	6	2.52 (0.41)	4.63 (0.56)	5	2.57 (0.19)	8.99 (0.89)
Mean		6.76 (0.51)	2.86 (0.33)		4.22 (0.90)	4.95 (0.74)

significantly less necrosis damage than leaves found in the forest understory (median = 3.77) ($U = 230.0$, $P = 0.002$, $r = 0.38$).

In this study, we found that leaf necrosis damage by the fungal pathogen *Calonectria* sp. decreased as anthocyanin content in young leaves increased, supporting the hypothesis that anthocyanin pigments may act as an anti-fungal agent to protect young developing leaves in the tropics (Coley & Aide 1989). In the tropics, almost 70 percent of a leaf's lifetime damage occurs during the small window of leaf expansion when young leaves lack developed cuticles and lignified cell walls (Coley & Aide 1989, Coley & Barone 1996). All plants encounter numerous pathogens in a natural environment and are known to produce secondary metabolites such as phenols, tannins, and flavonoids, to limit or inhibit pathogen attack (Lattanzio *et al.* 2006). Previous research has shown that anthocyanins—which are secondary metabolites belonging to the parent group flavonoids—reduce susceptibility to fungal pathogens in fruits and vegetative tissues (Schaefer *et al.* 2008, Hafidh *et al.* 2011). For example, fruit-rot in grape varieties infected with the necrotrophic fungus *Botrytis cinerea* was lower for grapes with high concentrations of anthocyanin compared to grapes with low anthocyanin concentrations (Schaefer *et al.* 2008), and anthocyanin extracts of red cabbage leaves inhibited fungal mycelial growth in *in vitro* bioassays (Hafidh *et al.* 2011). Red coloration by anthocyanin pigments could also provide a similar defensive function in developing young leaves when the risk of pathogen attack is high (García-Guzmán & Dirzo 2001). Our results, illustrated by Fig. 1, also show a similar pattern found by Schaefer *et al.* (2008) in grapes with high anthocyanin pigments, namely that the relationship between anthocyanin concentrations and fungal concentration was a negative exponential rather than linear. This may indicate that high anthocyanin content in young leaves is sufficient defense against pathogen attack; however, the broad range of damage in leaves with low anthocyanin content indicates that leaves use alternative anti-fungal compounds for defense.

We found significantly lower levels of necrosis damage to young leaves in the forest canopy relative to the understory. Canopy leaves are smaller, tougher and have higher phenolic contents relative to understory leaves (Coley & Barone 1996) which may make it harder for fungal pathogens to enter and extend throughout the leaf tissue. Moreover, fungal pathogen infection relies on moisture, favorable temperatures, and high relative humidity (Coley & Barone 1996), environmental conditions often lacking in the generally hot and dry forest canopy. Our results are in contrast to findings by García-Guzmán and Dirzo (2004) and Gilbert (1995) which found higher incidences of fungal disease symptoms in the canopy compared to the forest understory. One explanation from Gilbert (1995) was that higher incidences of canopy herbivory create leaf wounds that make canopy leaves more susceptible to pathogens. García-Guzmán and Heil (2013) observed that the pattern of fungal incidence between the forest understory and canopy changes when separating biotrophic from necrotrophic fungal pathogens. Necrotrophic pathogens are more commonly reported to infect shade-tolerant plants in the understory (García-Guzmán & Heil 2013), and our findings with necrotrophic *Calonectria* sp. are in line with this general pattern.

Leaves in the canopy also contain high levels of phenolic compounds (Coley & Barone 1996). Phenolic compounds such as anthocyanins may be present in higher concentrations in the forest canopy relative to the understory, but in this study there was no significant difference in anthocyanin content between canopy and understory leaves. These results are similar to those found by Dominy *et al.* (2002), which found that the canopy had no greater tendency to flush red than did the understory trees.

We found support for the hypothesis that fungal pathogen damage is negatively associated with anthocyanin content in young tropical leaves, but there are a few caveats. First, our study used a single generalist fungal pathogen, which cannot represent the wide variety of fungal pathogens in a tropical forest. Second, we surveyed a relatively small number of tree species in Parque Nacional San Lorenzo. Third, we did not directly test the toxicity of anthocyanins on our fungal pathogen. Last, we only measured anthocyanin pigments having a 525 nm or greater absorption wavelength. This may not be representative of the diverse group of anthocyanin pigments found within leaves that contribute to leaf defense. Further research testing how anthocyanins affect fungal pathogens must expand to include a wider range of fungal pathogens, including host-specific species, as well as a greater selection of tree species. Moreover, to examine whether resistance to fungi is caused by anthocyanins rather than other leaf properties, anthocyanins alone could be tested on various fungi grown *in vitro*. Despite these limitations, our study expands our under-

standing of the role that anthocyanins play in influencing plant-fungal interactions and provides greater insight into the selective factors for red leaf coloration in the humid tropics.

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