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Running title: Seed dormancy-defense syndromes.

Abstract. Seeds of tropical pioneer trees have chemical and physical characteristics that determine their capacity to persist in the soil seed bank. These traits allow seeds to survive in the soil despite diverse predators and pathogens, and to germinate and recruit even decades after dispersal. Defenses in seedlings and adult plants often are described in terms of trade-offs between chemical and physical defense, but the interplay of defensive strategies has been evaluated only rarely for seeds. Here we evaluated whether classes of seed defenses were negatively correlated across species (consistent with trade-offs in defense strategies), or whether groups of traits formed associations across species (consistent with seed defense syndromes). Using 16 of the most common pioneer tree species in a neotropical lowland forest in Panama we investigated relationships among four physical traits (seed fracture resistance, seed coat thickness, seed permeability, and seed mass) and two chemical traits (number of phenolic compounds and phenolic peak area), and their association with seed persistence. In addition, seed toxicity was assessed with bioassays in which we evaluated the activity of seed extracts against representative fungal pathogens and a model invertebrate. We did not find univariate trade-offs between chemical and physical defenses. Instead, we found that seed permeability – a trait that distinguishes physical dormancy from other dormancy types – was positively associated with chemical defense traits and negatively associated with physical defense traits. Using a linear discriminant analysis and a hierarchical cluster analysis we found evidence to distinguish three distinct seed defense syndromes that correspond directly with seed dormancy classes (i.e., quiescent, physical, and physiological). Our data suggest that short and long-term persistence of seeds can be achieved via two strategies: having permeable seeds that are well defended chemically, corresponding to the physiologically dormant defense syndrome; or having impermeable seeds that are well defended physically, corresponding to the physically dormant defense syndrome. In turn, transient seeds appear to have a lower degree of chemical and physical defenses,

corresponding to the quiescent defense syndrome. Overall, we find that seed defense and seed dormancy are linked, suggesting that environmental pressures on seed persistence and for delayed germination can select for trait combinations defining distinct dormancy-defense syndromes.

Keywords: Barro Colorado Island; dormancy types; pioneer trees; lowland tropical forests; plant defense theory; seed defenses; seed persistence; soil seed bank.

INTRODUCTION

Interactions between plants and their enemies profoundly influence ecological processes with implications that scale from local to global effects. For instance, consumption of plants by herbivores is one of the major paths for energy flow from autotrophs to the rest of the food web (Agrawal 2007). Herbivores consume and pathogens kill a considerable percentage of young plants (Agrawal 2010), playing a fundamental role in the maintenance of biodiversity (Fine et al. 2004, Bagchi et al. 2014). As a consequence, plants have evolved a wide range of chemical and physical defenses to limit damage by herbivores and pathogens (Agrawal and Fishbein 2006, Moles et al. 2013).

Plant defenses can be metabolically costly (Coley et al. 1985, Strauss et al. 2002). Nutrient limitation often shapes the balance in allocation among growth, reproduction, and defense (Coley et al. 1985, Agrawal 2007). The assumption that plant defenses are costly and the pool of resources is finite underlies theories to explain the distribution of defenses in plants (Moles et al. 2013). If plant defensive traits are equivalent in their effectiveness against enemies, defenses are predicted to trade off against one another (Koricheva 2002). Another possibility that is not mutually exclusive with the trade-off concept is the existence of plant defense syndromes (Kursar and Coley 2003, Agrawal and Fishbein 2006). In this framework, plants display a co-adapted complex of defensive traits forming consistent associations across species. The occurrence of defense allocation trade-offs or physical and chemical defense

syndromes has been evaluated on leaves of *Asclepias* spp (Agrawal and Fishbein 2006), and 261 different species of plants representing a broad taxonomic and geographic scope (Moles et al. 2013), without yielding consistent support for their existence (Agrawal 2007).

To date, explorations of plant defense theory have focused almost entirely on established plants, with little attention to seeds. Seeds are one the most important components of fitness for most flowering plants, leaving a critical gap in our current understanding of plant defense strategies (Dalling et al. 2011, Tiansawat et al. 2014). Seeds normally are defended by physical barriers and/or chemical compounds (Hendry et al. 1994, Davis et al. 2008, Tiansawat et al. 2014, Zalamea et al. 2015, Gripenberg et al. 2018), yet it remains unclear whether these defenses trade off with one another or instead comprise suites of traits that associate together across plant species (but see Davis et al. 2016).

After dispersal, seeds can either germinate or persist in the soil. Seed persistence, defined as survival until germination, increases the likelihood that some seeds will encounter favorable conditions for seedling recruitment (Long et al. 2015). Thus, understanding seed defenses requires attention to how, and for how long, seeds survive in the soil. Seeds can persist in the soil seed bank as a result of dormancy, where physical or physiological characteristics of the seeds prevent germination, or as a result of quiescence, where seeds have no barriers to prevent germination and germinate as soon as conditions become favorable (Thompson 2000, Dalling et al. 2011). Two of the major seed dormancy types, physical and physiological dormancy, are predicted to be functionally equivalent at delaying germination to avoid adverse environmental conditions for seedling recruitment and growth (Thompson 2000). It has also been assumed that the adaptive significance of seed dormancy is unrelated to defense traits against natural enemies. An alternative view is that defense traits and dormancy types are linked, and thus must be related to seed persistence. For instance, seed permeability, a trait that distinguishes physically dormant seeds from seeds of other

dormancy types (Baskin et al. 2000), can play a key role at determining attractiveness or accessibility of seed contents to granivores and pathogens (Paulsen et al. 2013, Zalamea et al. 2015).

Dalling et al. (2011) proposed that the three major types of persistent seeds commonly found in soil (i.e., physically dormant, physiologically dormant, and quiescent) rely on distinct sets of defenses, resulting in *seed dormancy-defense syndromes,* analogous to plant defense syndromes (Agrawal and Fishbein 2006). They predicted that seeds with physical dormancy rely on physical defenses to exclude enemies, whereas seeds with physiological dormancy deploy a continuum of physical and chemical defenses to deter enemies, and quiescent seeds depend on protection from seed-inhabiting microbes (Dalling et al. 2011).

We examined how physical and chemical defense traits of seeds are related to each other, and to seed persistence in the soil, in 16 of the most common pioneer tree species in lowland forest in Panama. We evaluated whether individual defensive traits of seeds are negatively correlated (consistent with the concept of univariate trade-offs in defense strategies), or whether traits form associations across species (consistent with seed defense syndromes). We further explored whether investment in different defense traits was consistently associated with interspecific variation in the capacity of seeds to persist in the soil.

Unlike the seeds of shade-tolerant species, which in moist tropical forests often germinate immediately, the seeds of pioneer trees mostly form seed banks. These seeds persist for different periods of time, and represent different dormancy types (Dalling et al. 1997, 1998a), making them ideal to test for the existence of *seed dormancy-defense syndromes.* Convergent evolution in dormancy classes across seed plants has been documented recently (i.e., evidenced by a large degree of homoplasy on the Spermatophyta phylogeny), and it has been proposed that seed dormancy can be evolutionarily labile (Willis

et al. 2014). However, the distribution of dormancy classes across the seed plant phylogeny is not random (Willis et al. 2014). Thus, to place seed defensive traits within an evolutionary context, we tested for congruence between the phylogenetic placement of the 16 study species and a classification of the species based on seed defensive traits. Finally, in addition to direct measurements of physical and chemical seed traits, we evaluated the seed toxicity directly for a subset of species through bioassays of seed extracts with a model invertebrate used widely in toxicology studies, and with two fungal pathogens.

METHODS

Study site and species

The study was carried out in seasonally moist lowland tropical forest at Barro Colorado Island, Panama (BCI; 9°10'N, 79°51'W). Rainfall on BCI averages 2600 mm yr^{-1}, with a pronounced dry season from January to April (Windsor 1990). We selected 16 of the most common pioneer tree species from lowland tropical forests in Panama, which recruit into gaps and other canopy openings (Table 1) (Condit et al. 1996, Dalling et al. 1998a,b). Seeds of neotropical pioneer species vary widely in dormancy type (Sautu et al. 2007), in size, and in their ability to persist in the soil (Dalling et al. 1997). The selected species are phylogenetically, morphologically, and functionally diverse, allowing us to examine the pioneer community's functional traits related to defense (Table 1). Here *Trema micrantha* (*sensu lato*) is considered to represent two species (Yesson et al. 2004): *Trema micrantha* "brown" is restricted to landslides and road embankments, while *Trema micrantha* "black" occurs mostly in treefall gaps (Silvera et al. 2003, Yesson et al. 2004, Pizano et al. 2011).

Seeds were collected from ripe fruits on the Barro Colorado Nature Monument (BCNM) in central Panama. After collection, seeds were allocated to i) measurements of seed defensive traits, ii) evaluation of chemical defenses via bioassays and iii) use in a burial experiment to determine seed persistence in the soil, as described below. The number or dry

151 weight of seeds used varied among species and traits (Table 1).

152 *Seed traits*

153 *Seed physical protection.* – Quantitative differences among species in physical
154 protection were represented by four traits: seed fracture resistance, seed coat thickness, seed
155 coat permeability, and seed mass. Seed fracture resistance and seed coat thickness are directly
156 related to seed toughness. Tougher seeds are less likely to be attacked by predators because
157 they are more energetically costly per unit of reward than weaker seeds (Fricke and Wright
158 2016). Seed coat permeability is a physical trait that distinguishes species with physical
159 dormancy from other dormancy types, and appears to be relevant to the potential for seeds to
160 be colonized by microbes from soil (Dalling et al. 2011, Zalamea et al. 2015). We included
161 seed mass as a physical trait, because it has been suggested that seed mass can be used as a
162 proxy of seed toughness (Fricke and Wright 2016). Prior to evaluation of physical defenses,
163 seeds were inspected, and those that showed cracks or anomalies on the seed surface were
164 discarded.

165 *Seed fracture resistance* was defined as the minimum force (N) required to initiate seed
166 rupture, as measured by an Instron Single Column Testing System Model 3342 (Instron
167 Company, USA). Each seed was loaded between the anvil and the compression probe and
168 then compressed until the seed coat ruptured. The seed coat rupture creates a sudden drop in
169 force, such that the instrument can precisely record the force causing the fracture.

170 *Seed coat thickness* was measured as the mean seed coat thickness (μm) for each seed.
171 Following Zalamea et al. (2015), seeds of each species were cut in half under a dissecting
172 scope and scanned using a Zeiss – Evo 40 vp scanning electron microscope. Mean seed coat
173 thickness was determined from measurements at four random points for each seed's image
174 via ImageJ (http://rsbweb.nih.gov/ij/).

175 *Seed coat permeability* was measured by fluorescent dye uptake into the endosperm.

Following Zalamea et al. (2015), seeds were incubated in 0.1% (w/v) aqueous solution of Lucifer yellow CH potassium salt (hereafter LY; Biotium, Inc., CA, USA) for 48 h in the dark at room temperature (22˚C). LY has a low molecular weight compared to other water-soluble fluorophores, making it especially useful for measuring seed permeability (Tieu and Egerton-Warburton 2000). After incubation, seeds were cut in half and examined using a Nikon Eclipse 600 microscope attached to a XX-V mercury lamp, with a Nikon B-2A fluorescent filter set (450–490 nm excitation/515 nm emission). Permeability was scored as zero (no LY in the endosperm) or one (LY in the endosperm).

Seed mass (mg) was measured with an analytical balance precise to ± 0.001 g. Seeds were removed from fruits and cleaned manually to remove fruit pulp or cottony filaments. Clean seeds were air-dried at room temperature (~22˚C) in the dark for ≥ 7 days, as needed for each species. A subsample of seeds was weighed several times prior to measurement to assure constant weight, indicative of dry seeds.

Seed chemical protection. – We focused on characterizing phenols to minimize the risk of confounding defensive and non-defensive compounds. Although phenolic compunds may have some non-defensive roles, they are known to protect plants and seeds from enemies such as seed predators and pathogens (Hendry et al. 1994, Davis et al. 2008, 2016, Gripenberg et al. 2018). Whole seeds were ground to a fine homogenate using a Wiley Mini Mill (model 3383-L10 Thomas Scientific, USA). For each species, three replicates of 0.1 g of ground seeds were extracted in methanol following Tiansawat et al. (2014) and Davis et al. (2016). The triplicates of methanol supernatant were then analyzed with high performance liquid chromatography (HPLC). HPLC measurements of total phenol content were made following Gallagher et al. (2010) at the USDA-ARS National Center for Agricultural Utilization Research (Peoria, Illinois, USA). We focused our analysis on the non-volatile fractions of seed homogenates extracted using methanol/DMSO (dimethyl sulfoxide), to

ensure comprehensive profiles of phenolic compounds that were comparable among species.

Phenolic peak area (the area of each peak in each sample) was first standardized to the mass of the seed sample to allow us to compare compound abundance across species:

$$Mass\text{-}standardized\ peak\ area = \frac{\frac{Raw\ peak\ area}{Injection\ volume} \times Total\ volume\ of\ extract}{Sample\ mass}$$

where peak area was measured in mV x min, injection volume was 25 mL, extract volume was 1.5 mL, and sample mass was measured in g. The mass-standardized peak area of all potential defensive compounds present in each sample was then summed and mean peak areas were calculated from three replicates per species.

Phenolic compounds, or the number of absorbance peaks of potential defensive compounds, were distinguished according to their retention times. Peak detection was set at 280 nm.

Functionally relevant chemical defenses

Seed toxicity was assessed through bioassays of seed extracts with a model organism used widely in toxicology studies, brine shrimp (*Artemia franciscana*), and two fungal pathogens (*Fusarium* sp. 1 and *Fusarium* sp. 2) isolated from seeds of pioneer trees (*Hieronyma alchorneoides* and *Trema micrantha* "black") that were buried and retrieved from a common garden experiment on BCI (Sarmiento et al. 2017). Detailed protocols for preparing the seed extracts and performing the bioassays are presented in online supporting information (Appendix S1).

Brine shrimp were hatched in a 2L tank under constant light and aeration. After two days, larvae were removed from the tank for use in bioassays. Seed homogenates of 14 out of the 16 species used previously were de-fatted, extracted with methanol, and allowed to dry in a fume hood. The remaining pellet was re-suspended in distilled water, in combination with a

prepared salt-water aquarium mixture, to create a range of concentrations of seed extract. Test tubes containing 10 larvae and the aqueous solution with different concentrations of seed extract (0 $\mu g\ mL^{-1}$ (control), 1 $\mu g\ mL^{-1}$, 5 $\mu g\ mL^{-1}$, 10 $\mu g\ mL^{-1}$, 100 $\mu g\ mL^{-1}$) were included in four replicate blocks. Dead larvae were counted after 24 hours.

Species of *Fusarium* are important seed pathogens (Agrawal and Sinclair 1996) and commonly infect seeds of pioneer species in lowland tropical forests (Gallery et al. 2007, Shaffer et al. 2016, Sarmiento et al. 2017). Based on seed availability, we selected a subgroup of 10 tree species for the fungal bioassays. Sterile 96-well plates were used to measure fungal growth *in vitro* following a treatment with 1:1 w:v dilution of seed extracts prepared with sterile distilled water. Wells containing seed extracts were inoculated with fungi, wells that only had seed extracts were used as negative controls, and wells that contained sterile water were inoculated with fungi and used as positive controls. Plates were incubated at room temperature (~25˚C). Immediately after inoculation initial readings of cell density were made at 750nm. Thereafter, each plate was sealed with Parafilm and placed in a plastic bag with damp paper towels to keep moist. Readings were repeated every 3 days for a total of 15 days. Spectrophotometer readings for wells with extracts and fungi were scaled by the mean value of negative controls (i.e., seed extracts alone). A fungal growth index was calculated as cell density after 15 days divided by cell density on the positive control on each plate.

Seed persistence in the soil

To determine the rate at which seed viability declines in soil over time when exposed to natural abiotic and biotic factors such as microbes, but excluding seed predators, we conducted a seed burial experiment in the forest at BCI. Seeds were removed from ripe fruits collected from the canopy of, or the ground beneath, at least five fruiting trees of each of the 16 species at Barro Colorado Nature Monument. Seeds from all maternal sources of each species were pooled and 200 seeds from this pool were placed in a germination experiment to

249 estimate initial seed viability. Another 900 seeds from the same pool for each species were
250 used in the seed burial experiment.
251 The burial experiment was initiated in February 2012. Seeds from each species were
252 buried in small mesh bags beneath the mature forest canopy in five 9 x 15 m common
253 gardens on BCI (Zalamea et al. 2015, Ruzi et al. 2017, Sarmiento et al. 2017). We used a
254 randomized complete block design, with gardens in multiple soil types (Baillie et al. 2007;
255 BCI soil map: http://strimaps.si.edu/webmaps/bcnm/). To avoid germination, gardens were
256 located in the understory and in areas that contained no adults of the study species within 20
257 m of the garden edges. Twenty seed bags per species were prepared. Each consisted of 45
258 seeds of one species mixed with 10 g of sterile forest soil (autoclaved previously for 2 h at
259 121°C), enclosed in a nylon mesh bag (pore size = 0.2 mm), and covered with an aluminum
260 mesh (pore size = 2 mm). Four bags per species were buried in each garden at a depth of 2
261 cm below the soil surface.
262 Seed bags were recovered from the gardens 30 months after burial. After recovery, seed
263 bag contents were emptied into a sieve, and seeds were retrieved after rinsing with tap water.
264 To record germination, 10 seeds per seed bag (or fewer, if seeds decayed in the bags) were
265 selected randomly and placed in a Petri dish lined with a paper towel, moistened with sterile
266 distilled water, sealed with 2 layers of Parafilm®, and incubated for 6 weeks in a shadehouse
267 on BCI under 30% full sun, high red:far-red irradiance (ca. 1.4), and ambient temperature.
268 The maximum temperature recorded on the germination bench was ca. 38°C (Zalamea et al.
269 2015), similar to the temperature near the soil surface in large treefall gaps on BCI (Marthews
270 et al. 2008).
271 Germination was defined as radicle protrusion and was recorded weekly for six weeks.
272 Fresh and buried seeds that did not germinate after six weeks were assessed for viability
273 using the tetrazolium test (TZ). This test is based on the activity of dehydrogenase enzymes

that reduce the 2, 3, 5-triphenyl tetrazolium chloride in the living tissues (Peters 2000). Ungerminated seeds scored as viable by TZ testing were considered dormant, and total viability was calculated as the sum of germinated and dormant seeds. Seed persistence was then calculated as the proportion of initially viable seeds that survived after 30 months of burial.

Data analysis

We assessed pairwise Pearson correlations among all traits to determine whether seed defense traits exhibited univariate trade-offs. Significance was determined after a Bonferroni correction and results are shown in the supporting information. To account for non-independence of seed traits within related plant lineages, phylogenetic independent contrasts (PIC) (Felsenstein 1985) were calculated for each trait using the *pic* function in the package *ape* (Paradis et al. 2004) in R (R Development Core Team 2017), using a previously constructed phylogeny (Webb and Donoghue 2005). All pairwise correlations of PICs also were calculated. Mean values for each seed trait used in this study were calculated from a variable number of seeds (Table 1), and all values were log-transformed before analysis. To ensure finite and non-zero values in the dataset, a small adjustment of 0.001 was used for proportion data (i.e., seed permeability and persistence) and an adjustment of 1 was used for the number of phenolic compounds and the phenolic peak area.

Seeds of tropical pioneer trees usually form seed banks in which they persist until conditions are adequate for germination. To do so they use different dormancy strategies (Dalling et al. 1997, 2011). Seed permeability is a key trait that distinguishes physical dormancy from other dormancy types. It also has been suggested to affect accessibility of seed contents to granivores and/or pathogens (Paulsen et al. 2013, Zalamea et al. 2015). To examine relationships between seed persistence, as well as seed permeability, and the physical and chemical defenses of seeds, we used a structural equation model (SEM)

299 approach and tested 15 different models including all the measured and latent variables. We
300 selected the model based on minimization of the Akaike information criterion (AIC) and
301 likelihood ratio tests (Burnham and Anderson 2002).
302 To explore the existence of seed defense syndromes we used a linear discriminant
303 analysis (LDA) of seed syndrome group, defined as the seed dormancy type (i.e., physical,
304 physiological, or quiescent) suggested by Dalling et al. (2011), against seed defensive traits
305 and performed a k-means classification of the resulting scores. For testing the congruence
306 between the species phylogeny and the trait-based classification, we used the phylogenetic
307 tree that was constructed previously to calculate PICs. For the phenogram, we performed a
308 hierarchical cluster analysis based on Euclidean distances and the Ward's linkage method,
309 following Becerra (1997) and Agrawal and Fishbein (2006). To have comparable measures
310 among traits, our physical and chemical mean trait values were previously transformed to Z
311 scores (mean = 0, SD = 1). To test congruence between the phylogenetic tree and the
312 phenogram, we calculated pairwise distance matrices between the pairs of tips as the branch
313 length for the phylogenetic tree using the function *cophenetic.phylo* and as the Euclidean
314 distance between tips of the phenogram. The correlation between the two matrices was
315 determined by a Mantel test via the *mantel.rtest* function in R.
316 RESULTS
317 Five of 15 pairwise correlations between physical traits (i.e., seed fracture resistance,
318 coat thickness, permeability and mass) and chemical traits (i.e., phenolic peak area and
319 number of phenolic compounds) were significant after accounting for phylogenetic non-
320 independence of these traits within related plant lineages (Table 2). Without accounting for
321 phylogenetic non-independence, we found that 6 of 15 pairwise correlations between physical
322 traits and chemical traits were significant (Appendix S2: Table S1). All significant
323 correlations between seed defensive traits were positive, with or without phylogenetic

correction, suggesting that none of these univariate relationships was consistent with the trade-off concept between seed defenses.

After controlling for phylogeny, only 2 of 18 possible correlations between physical or chemical traits and the secondary metabolite bioassays were significant (Table 2). In addition, results of the fungal pathogen assays were not correlated with seed toxicity to invertebrates, as represented by the brine shrimp bioassay (Table 2, Appendix S2: Table S1). Results of the two fungal assays were highly correlated with one another (Table 2).

The proportion of initially viable seeds that survived after 30 months of burial (i.e., seed persistence) varied widely among species (Table 1). Seed persistence ranged from 8% for *Cochlospermum vitifolium* to 100% for *Annona spraguei*, *Zanthoxylum ekmanii* and *Trema micrantha* "brown". No PIC correlations between seed persistence and physical traits was significant. However, PIC correlations between seed persistence and the abundance of phenolic compounds, as well as seed persistence and seed toxicity to invertebrates, were positively correlated (Table 2). Finally, we found a marginally significant and positive PIC correlation between seed persistence and the number of phenols. These results are consistent with the SEM results showing that chemical defenses are positively associated with seed persistence, while physical defenses are not (see below).

Seeds of pioneer trees have different dormancy strategies that allow them to persist in the soil seed bank, and seed permeability is one of the well-known traits used to distinguish physically dormant seeds from other seeds. Here, we used SEMs to quantify possible associations among seed physical defensive traits (i.e., seed fracture resistance, seed coat thickness, and seed mass), seed chemical defenses (i.e., number of phenolic compounds and phenolic peak area), and seed persistence and permeability. Model selection strongly indicated that physical defenses are negatively associated with seed permeability and not associated with seed persistence, and that chemical defenses are positively associated with

seed permeability and seed persistence (Appendix S2: Table S2). The SEM that best explained the associations between seed persistence and permeability with seed physical and chemical defenses retained the latent variable "physical 2" (consisting of seed fracture resistance and seed mass), and the number of phenolic compounds (Fig. 1a; Model 5 in Appendix S2: Table S2, $p < 0.001$, AIC = 190.6, Akaike weight = 0.64) and explained 55% of variation in seed permeability and 19% of variation in seed persistence. The second best-supported model retained the latent variable "physical 2, and the phenolic peak area (Fig. 1b; Model 6 in Appendix S2: Table S2, $p < 0.001$, AIC = 191.9, Akaike weight = 0.34) and explained 31% of variation in seed permeability and 29% of variation in seed persistence.

A linear discriminant analysis (LDA) of seed dormancy types against seed defensive traits (i.e., 4 physical and 2 chemical traits) revealed that 80% of variance was explained by the first linear discriminator (LD1), and 20% explained by the next (LD2). The first linear discriminator was negatively associated with seed fracture resistance and coat thickness, and positively associated with seed permeability, seed mass, phenolic peak area, and number of phenolic compounds. The second discriminator was negatively associated with seed coat thickness, permeability, seed mass and phenolic peak area, and positively associated with seed fracture resistance and number of phenolic compounds. In addition, the first linear discriminator separated two groups of species: one consisting of species with physically dormant seeds, and the other consisting of species with physiologically dormant and quiescent seeds (Fig. 2). In turn the second linear discriminator separated physiologically dormant from quiescent seeds (Fig. 2). The k-means classification for all of the 16 species used in this study confirmed the existence of three groups of species consistent with seed dormancy types. When the dataset was split in half with training, the model was again 100% accurate in distinguishing between the three groups. In summary, seeds from the physically dormant defense syndrome have impermeable seeds that are mainly defended by physical

barriers. Seeds from the physiologically dormant defense syndrome have permeable seeds heavily defended by the presence and abundance of phenolic compounds. Seeds from the quiescent defense syndrome have a lower degree of chemical and physical defenses. When more traits were included in the analysis (i.e., seed persistence and chemical defense bioassays), the species classification into groups mirrored seed dormancy types (Appendix S2: Table S3). This is true even if including other traits resulted in a reduction in the number of species to 15 when seed persistence was included, 13 when the invertebrate assay was included and 9 when the fungal pathogen assays were included (Appendix S2: Table S3).

Similarly, hierarchical cluster analysis of chemical and physical defense traits revealed three distinct groups (Fig. 3). These three groups are consistent with those designated by the LDA and highlight three seed dormancy-defense syndromes. As in the LDA results, quiescent and physiologically dormant seeds are more similar to each other than to physically dormant seeds. The only exception between the results from the hierarchical cluster analysis and the LDA was *Trema micrantha* "black", which was classified in different groups (Fig. 3). Overall, phylogenetic relationships are correlated with the defense trait cluster (Mantel $r = 0.44$, $p < 0.001$), suggesting that the seed dormancy-defense syndromes are at least in part constrained by phylogenetic relationships.

DISCUSSION

We found no evidence of direct univariate trade-offs between chemical and physical defenses of seeds for a phylogenetically broad group of tropical pioneer trees. Our results do not support redundancy in the context of univariate trade-offs between chemical and physical defenses, in which it is suggested that if one defense is sufficient to deter herbivores and/or pathogens, selection against redundant defenses should be strong (see Agrawal 2007, 2010). Instead, we found strong evidence suggesting that seed permeability is positively associated

with chemical defenses, and negatively associated with physical defenses, which represents an indirect trade-off mediated by seed permeability.

Studies using seedlings and adult plants have found mixed evidence for trade-offs between physical and chemical defenses. For instance, Twigg and Socha (1996) found strong negative correlations between physical deterrents and fluoroacetate concentration in fresh leaves of four species of *Gastrolobium*. In contrast, analysis of defensive traits of leaves from 24 different species of *Asclepias* revealed few significant, but all positive correlations among defensive traits (Agrawal and Fishbein 2006). A recent study focusing on physical and chemical defenses in diverse plants across a large geographic scope consistently found no evidence for trade-offs between physical and chemical defenses (Moles et al. 2013). The few studies focused on seeds do not escape this debate: there is evidence supporting (Zhang et al. 2016) or rejecting (Tiansawat et al. 2014) the univariate trade-off model between physical and chemical defenses (see also Gripenberg et al. 2018).

The absence of univariate trade-offs does not mean that trade-offs are not important in explaining seed defense strategies. In nature, plants are expected to allocate resources to several defensive traits simultaneously (Agrawal and Fishbein 2006). The absence of consensus around the univariate trade-off model could reflect evolution toward convergent defense syndromes, in which a co-adapted complex of traits form associations across species (see Kursar and Coley 2003, Agrawal 2007), and these different groups of traits negatively covary.

Dormancy-defense syndromes in tropical pioneer seeds

In the absence of evidence for univariate trade-offs between physical and chemical defenses, alternative frameworks have gained popularity for understanding the evolution of suites of traits that can lead to defense syndromes (Kursar and Coley 2003, Agrawal and Fishbein 2006). Dalling et al. (2011) hypothesized that selection on seed dormancy and

resistance to enemies should result in distinct dormancy-defense syndromes. In part this reflects the observation that seeds can persist in the soil seed bank as a result of dormancy or quiescence (Baskin and Baskin 2004, Dalling et al. 2011). Dormant seeds can have physical or physiological barriers to avoid germination under unfavorable conditions while quiescent seeds do not have such barriers and germinate when conditions are favorable (Dalling et al. 2011). Here, we revealed three distinctive groups of species defined by physical and chemical traits of seeds. As proposed by Dalling et al. (2011) and suggested for temperate species by Davis et al. (2016), these groups of species strongly support the existence of dormancy-defense syndromes for 16 of the most common species of pioneer trees in the focal lowland tropical forest.

Dormancy-defense syndromes can be informed by placing seed traits in an evolutionary context. Correlation between the trait phenogram and the plant species phylogeny suggests that at least in part seed dormancy-defense syndromes track phylogenetic history. In our study, species with physically dormant seeds are primarily within the Malvales, likely influencing the significant correlation between the defense phenogram and the species phylogeny. Although the proportion of species with physically dormant seeds on the Spermatophyta phylogeny is smaller compared to other dormancy classes, our results are in agreement with other studies that focused on the evolution of seed dormancy and found that the distribution of dormancy classes across the phylogeny is not random (Willis et al. 2014).

How do seeds of tropical pioneers persist in the soil seed bank?

Seed persistence in the soil impacts tree species abundance and distribution by determining when and where seeds can germinate (Long et al. 2015). Although all species included in this study can persist to some degree in the soil seed bank, we found that seed mortality rates varied greatly among species. For instance, after 30 months of soil incubation, 92% of initially viable seeds of *Cochlospermum vitifolium* died, but seed mortality was 0%

448 for *Annona spraguei*, *Zanthoxylum ekmannii*, and *Trema micrantha* "brown". In a review
449 about seed persistence Long et al. (2015) classified persistence in the soil seed bank as
450 transient (surviving less than a year), short-lived (surviving between 1 and 3 years) or long-
451 lived (surviving more than 3 years). Although we did not include incubation times longer
452 than 30 months, some of the species showed no decrease at all in seed viability suggesting
453 that we sampled species from all three persistence classes. Our results are also supported by
454 observations of naturally dispersed seeds of *Zanthoxylum ekmannii* and *Trema micrantha* that
455 have decades-long persistence in the soil on Barro Colorado Island (Dalling and Brown
456 2009).

457 Seed permeability is a trait commonly used to distinguish physically dormant seeds
458 from other seeds (Baskin et al. 2000) and it has been hypothesized as a key trait that
459 determines accessibility of seed contents to granivores and/or pathogens (Paulsen et al. 2013,
460 Zalamea et al. 2015). Although we did not find evidence for univariate trade-offs between
461 physical and chemical defenses, we found an indirect trade-off mediated by seed permeability
462 in which seeds that are defended by thick and strong physical barriers tend to be
463 impermeable, and seeds heavily defended by the presence and abundance of phenolic
464 compounds tend to be permeable. In temperate regions, long seed persistence is achieved
465 through investment in chemical defenses (Hendry et al. 1994) and in wet tropical habitats
466 where pathogen pressure is high, physical defenses alone may be insufficient to prevent
467 pathogen infection, thus investment in chemical defense may be a solution to achieve long
468 seed persistence. In a tropical montane forest in Costa Rica, morpho-physiological dormancy
469 allows seeds of *Bocconia frutescens* to achieve long persistence through chemical defenses
470 (Veldman et al. 2007). Although we did not find any significant relationship between the
471 abundance or presence of phenolic compounds and seed bioassays, using PIC correlations
472 and SEMs, we found that seed persistence was positively associated with the abundance of

phenolic compounds. In addition, we also found that seed toxicity in the invertebrate bioassay was positively correlated with seed persistence. These results strongly support the idea that long seed persistence in tropical forests can be achieved through investment in chemical defenses.

The observation that seed persistence was positively correlated with the invertebrate bioassay and negatively, but only marginally, correlated with one of the fungal bioassays, raises the possibility that the chemistry that impacts invertebrates does not necessarily reduce fungal growth and *vice versa*. However, an important caveat is the possibility that our fungal growth assays not only detect the effects of seed toxins, but also incorporate positive nutritional effects of seed tissue extracts on fungal growth. While chemical seed defenses might be expected to be concentrated in external seed coat or fruit tissues, seed extracts in our assays were prepared using whole seeds, reflecting the difficulty of separating the seed coat from interior nutrient-rich endosperm and embryo. Notably, the only species that consistently reduced fungal growth in comparison to controls was *Zanthoxylum ekmannii*, which had among the highest abundance and diversity of phenolic compounds. In contrast, *Cochlospermum vitifolium*, one of the most strongly physically-defended species, had almost twice the fungal growth on seed extracts compared to controls. Thus, more data are needed to clearly understand whether chemical defenses affect invertebrates and fungal pathogens differently.

Variable biotic and abiotic pressures may have selected for the evolution of different seed defense syndromes. Our results suggest that seeds of tropical pioneer trees can achieve short and/or long persistence using two different types of seeds: i) permeable seeds heavily defended by the presence and abundance of phenolic compounds (i.e., corresponding to the physiologically dormant defense syndrome), or ii) impermeable seeds that are mainly defended by physical barriers (i.e., corresponding to the physically dormant defense

syndrome). Weed seeds from temperate regions have solved the problem of persistence through similar approaches, but contrary to our results, seeds that attain long persistence rely more on physical defenses, whereas species with shorter persistence rely more on chemical defenses (Davis et al. 2016). This result suggests that species could vary considerably in resource allocation to defense among different habitats. Finally, we found evidence that seeds of quiescent species persist in the soil for short periods of time, lacking or having greatly diminished chemical and physical defenses (i.e., corresponding to the quiescent defense syndrome). These seeds may be especially dependent upon protection gained from beneficial seed-inhabiting microbes, as suggested in previous studies (Gallery et al. 2010, Dalling et al. 2011, Sarmiento et al. 2017). However, to draw firm conclusions we need further studies testing the effect of inoculations on seed survival.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

671 TABLE 1. Characteristics of the study species, including dormancy type, persistence index, mean seed fracture resistance, mean seed coat
672 thickness, percentage of permeable seeds, mean seed mass, number of phenolic compounds, and total phenolic peak area. Seed chemical
673 defenses were tested against brine shrimp (*Artemia franciscana*) and two fungal isolates (*Fusarium* sp. 1 and *Fusarium* sp. 2). For seed fracture
674 resistance, seed coat thickness, and seed mass the standard deviation of the mean and number of seeds used to obtain species-specific mean trait
675 estimate are in parentheses.

Species	**Dormancy type**	**Persistence**	**Fracture resistance (N)**	**Coat thickness (µm)**	**Permeability (%)**	**Mass (mg)**	**Phenolic compounds (number of peaks)**	**Phenolic (peak area)**	**Brine shrimp ED50 (µg mL^{-1})**	***Fusarium* sp1**	***Fusarium* sp2**
Apeiba membranacea	physical	0.59	166.4 (87.7 ; 95)	121.6 (22.6 ; 34)	36	13.6 (1.2 ; 154)	0	0	3.03E-01	1.4	1.2
Cochlospermum vitifolium	physical	0.08	231.9 (55.5 ; 91)	178.9 (13.5 ; 32)	22	24.5 (2.2 ; 123)	1	313569320	4.38E-01	1.9	1.8
Colubrina glandulosa	physical	0.22	43.8 (7.9 ; 93)	198.3 (19.7 ; 19)	34	15.1 (1.7 ; 191)	4	122649420	-	-	-
Guazuma ulmifolia	physical	0.92	38.5 (10.9 ; 100)	94.2 (8.0 ; 17)	17	3.4 (0.3 ; 322)	2	985146508	6.77E-01	1.2	0.9
Luehea seemannii	physical	0.73	39.9 (23.5 ; 79)	46.5 (4.6 ; 40)	12	1.9 (0.3 ; 226)	0	0	1.95E+00	-	-
Ochroma pyramidale	physical	0.82	40.4 (6.6 ; 95)	132.6 (4.8 ; 40)	12	5.7 (0.3 ; 178)	0	0	1.07E+00	1.3	1.0
Annona spraguei	physiological	1	175.1 (95.6 ; 91)	72.2 (18.8 ; 40)	88	45.4 (3.0 ; 231)	12	3747381475	8.35E-01	1.3	1.5
Hieronyma alchorneoides	physiological	0.63	38.8 (7.9 ; 100)	287.8 (55.8 ; 35)	100	6.5 (0.3 ; 340)	24	2327874247	2.85E-01	1.3	1.2
Lindackeria laurina	physiological	-	11 (4.7 ; 50)	61.6 (10.3 ; 15)	90	74.3 (17.2 ; 170)	17	726948505	1.90E-01	-	-
Trema micrantha "black"	physiological	0.98	9.5 (2.6 ; 90)	101.3 (14.6 ; 39)	100	3.2 (0.2 ; 306)	1	367048261	9.76E-01	1.3	1.1
Zanthoxylum ekmanii	physiological	1	68.3 (24.3 ; 100)	268.8 (58.0 ; 35)	100	16.2 (0.8 ; 338)	13	917543536	3.13E+00	0.8	0.8
Cecropia insignis	quiescent	0.23	2.7 (0.9 ; 85)	40.6 (5.0 ; 30)	95	0.4 (0.03 ; 216)	1	108260613	1.00E+00	-	-
Cecropia longipes	quiescent	0.41	11 (5 ; 98)	24.8 (2.6 ; 30)	100	0.9 (0.07 ; 340)	3	56083277	1.18E-01	-	-
Cecropia peltata	quiescent	0.39	9 (4.4 ; 93)	29.7 (4.1 ; 30)	93	0.7 (0.06 ; 317)	1	278427782	1.22E+00	-	-
Ficus insipida	quiescent	0.45	9.8 (3.1 ; 100)	85.1 (12.5 ; 32)	93	1.6 (0.1 ; 340)	7	222405778	-	1.5	1.2
Trema micrantha "brown"	quiescent	1	11 (1.8 ; 85)	66.6 (6.6 ; 35)	99	1.7 (0.1 ; 204)	5	4563936147	4.29E+00	1.3	1.1

676

TABLE 2. Pairwise correlations using phylogenetic independent contrasts of seed persistence index, defense-related traits (i.e., physical and chemical traits), and seed bioassays (i.e., brine shrimp and *Fusarium* spp.) among pioneer tree species. The number of species used in each correlation is presented in parentheses.

		Physical traits				**Chemical traits**		**Bioassays**		
	Seed persistence	**Fracture resistance**	**Coat thickness**	**Permeability**	**Mass**	**Phenolic compounds**	**Phenolic peak area**	**Brine shrimp ED50**	***Fusarium* sp1**	***Fusarium* sp2**
Seed persistence	-	0.01 (15)	-0.03 (15)	0.12 (15)	0.06 (15)	0.18° (15)	**0.40** (15)**	**0.26* (13)**	-0.28° (10)	-0.08 (10)
Fracture resistance		-	0.09 (16)	-0.07 (16)	**0.33* (16)**	0.06 (16)	-0.02 (16)	-0.05 (14)	0.08 (10)	**0.43* (10)**
Coat thickness			-	0.06 (16)	**0.20* (16)**	**0.38* (16)**	0.14° (16)	-0.08 (14)	-0.09 (10)	-0.14 (10)
Permeability				-	-0.06 (16)	0.10 (16)	0.06 (16)	-0.06 (14)	-0.13 (10)	-0.14 (10)
Mass					-	**0.45** (16)**	0.13° (16)	-0.06 (14)	0.07 (10)	**0.53* (10)**
Phenolic compounds						-	**0.53** (16)**	-0.05 (14)	-0.09 (10)	-0.12 (10)
Phenolic peak area							-	-0.04 (14)	-0.11 (10)	0.09 (10)
Brine shrimp ED50								-	0.07 (9)	0.02 (9)
***Fusarium* sp1**									-	**0.70** (10)**
***Fusarium* sp2**										-

*** P < 0.05**

**** P < 0.01**

° P < 0.1

681 FIGURE LEGENDS

682 FIG. 1. Structural equation models that best explained the associations between seed
683 persistence and permeability, and seed physical and chemical defenses. (A) The model that
684 best explained the associations retained the latent variable "physical 2" (consisting of seed
685 fracture resistance and seed mass), and the number of phenolic compounds. (B) The second
686 best supported model retained the latent variable "physical 2", and the phenolic peak area.
687 Solid lines represent significant relationships and dotted lines represent non-significant
688 associations.

689 FIG. 2. Linear discriminant analysis (LDA) of seed dormancy syndromes against seed
690 defensive traits (i.e., 4 physical and 2 chemical traits). The first linear discriminator (LD1)
691 explained 80% of variance and LD2 explained 20%. Each point represents one of the species
692 included in the study. Three dormancy-defense syndromes were classified and colored as: i)
693 seeds that are mainly impermeable and defended by physical barriers, here denominated as
694 physically dormant group and colored in green, ii) permeable seeds that are heavily defended
695 by the presence and abundance of phenolic compounds, here denominated as physiologically
696 dormant group and colored in purple, and iii) seeds of species that have a lower degree of
697 chemical and physical defenses, here denominated as quiescent and colored in orange.

698 FIG. 3. Schematic comparison between the plant species phylogeny and the defense trait
699 cluster of 16 species of pioneer trees from central Panama. The hierarchical cluster analysis
700 of chemically and physically related traits revealed three distinct groups. Branches of these
701 three groups are colored in congruence to the three different dormancy-defense syndromes
702 found on the LDA, where seeds that are physically dormant are colored in green,
703 physiologically dormant in purple, and quiescent in orange.