

The interplay between propagule pressure, seed predation and ectomycorrhizal fungi in plant invasion

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Abstract

There are many hypotheses aiming to explain invasion success, but evaluating individual hypotheses in isolation may hinder our ability to understand why some species invade and others fail. Here we evaluate the interaction between propagule pressure, seed predation and missed mutualism in the invasion success of the pine, *Pinus ponderosa*. We evaluated the independent and interactive effects of propagule pressure and seed predation at increasing distances from a pine plantation. Additionally, because pines are obligate mutualists with ectomycorrhizal fungi (EMF) and pine invasions fail in the absence of their EMF symbionts, we evaluated EMF availability through a growth chamber bioassay. In this bioassay we measured root colonization by EMF with soil samples collected from the different distances from the plantation. We found that propagule pressure overwhelms seed predation only at the edge of the pine plantation, while seed predation overcomes propagule pressure at 25 m and further distances from the plantation. We also found that EMF root colonization decreases with distance from the plantation. However, pine roots were colonized up to 200 m from the plantation, suggesting that EMF may not be hindering invasion, at least not on the scale of this experiment. Taken together our results demonstrate that seed predation may be limiting the invasion of *P. ponderosa* in the study region as propagule pressure only overcomes seed predation at the plantation edge. Here we provide evidence of how strong biotic resistance can suppress an invasion, regardless of the variation in propagule pressure and the availability of mutualists.

Keywords

Biotic resistance, granivory, invasion mechanism, mutualism, *Pinus*, seed dispersal

Introduction

For decades, ecologists have tried to explain why some populations of introduced species become invasive while others fail to invade (Elton 1958, Lockwood et al. 2013). Numerous hypotheses seek to explain the mechanisms behind biological invasions (Catford et al. 2009). The propagule pressure hypothesis posits that increasing the number of individuals released in a region where they are not native increases the probability of invasion success (Lockwood et al. 2005). This hypothesis seems to be more supported by empirical evidence than most other hypotheses proposed in invasion biology (Simberloff 2009, Lamarque et al. 2011, Blackburn et al. 2015) and, therefore, may be the hypothesis carrying the greater consensus (Jeschke 2014). Another major hypothesis in biological invasions is the biotic resistance hypothesis (Elton 1958), which states that competitors, predators, herbivores and pathogens in the resident community reduce invasion success (Levine et al. 2004). Seed predation is one of the major filters for plant establishment (Janzen 1969, Hulme 1994, Larios et al. 2017) and, consequently, a major barrier to plant invasions (Nuñez et al. 2008, Pearson et al. 2011, Connolly et al. 2014). Studies on the interplay between propagule pressure and biotic resistance found that the influence of propagule pressure on invasion success is more important for high levels of biotic resistance (von Holle and Simberloff 2005, Jones et al. 2017).

Together with strong biotic resistance and low propagule pressure, the absence of mutualistic biotic interactions (missed mutualism) is one of the most important limitations for the invasion of non-native plants (Traveset and Richardson 2014, Dickie et al. 2017). More than one third of the most widespread invasive woody species form mutualistic interactions with mycorrhizal symbionts, more than two thirds form interactions with seed dispersers and the vast majority of invasive woody plants are animal pollinated (Traveset and Richardson 2014). If a non-native plant needs an obligate mutualist its absence in the invaded range will act as a strong filter to its invasion success (Traveset and Richardson 2014, Dickie et al. 2017). Even though the importance of different mechanisms is recognized in invasion processes, most studies on biological invasions focus on a single hypothesis without considering the possible interaction between different mechanisms. As a consequence, we lack an understanding of how different processes interplay to determine invasion success or failure (Pearson et al. 2018).

Pines are an ideal system to study the role of propagule pressure, seed predation and missed mutualism on invasion success. The role of propagule pressure can be easily studied because non-native pines produce a large number of seeds which are wind dispersed over large areas but with the highest proportion falling near the seed source, creating a gradient of propagule pressure (Nathan et al. 2011). The role of biotic resistance, through seed predation, can be assessed because seeds of non-native pines are highly consumed by native generalist seed predators (Nuñez et al. 2008, Chiuffo et al. 2018). Additionally, pines are obligate mutualists with ectomycorrhizal fungi (EMF) and pine invasion fails in the absence of their EMF symbionts (Nuñez et al. 2009), thus the effect of missing mutualism can be straightforwardly evaluated using pines. Furthermore, EMF are dispersed from the invasion source, creating a gradient of de-

creasing abundance of fungal symbionts (Collier and Bidartondo 2009, Hayward et al. 2015, Horton 2017).

The aim of this study was to test the importance of three mechanisms (propagule pressure, seed predation and missed mutualism) in the invasion of *Pinus ponderosa* in north Argentinean Patagonia. Here, we evaluated seed predation with different seed availability to resemble the natural seed rain pattern at different distances from a plantation of *P. ponderosa*. Additionally, to test if the absence of highly co-evolved soil mutualists could account for pine invasion failure we performed a growth chamber bioassay with soil inoculum from increasing distances from the pine plantation. Specifically, we asked the following question: what is more important determining *P. ponderosa* invasion success, propagule pressure, seed predation, the availability of EMF symbionts or the interplay between these mechanisms? Evaluating the relative importance of different invasion mechanisms is key to identify the causes of plant invasions. Also, assessing the interaction between different mechanisms helps to understand how different processes interplay to determine invasion success or failure (Pearson et al. 2018).

Methods

Study area

We conducted this experiment on a steppe ecosystem in North Patagonia (40°59'53"S, 71°05'13"W) because this type of ecosystem is among the most frequently invaded by non-native pines (Richardson et al. 1994). In Patagonia, large areas of steppe have been replaced by pine plantations during the past few decades (CIEFAP 2017), which are now seed source for invasions. *Pinus ponderosa* is the most widely planted species in the region, covering over 90% of the forestry area (CIEFAP 2017). However, *P. ponderosa* is only rarely invasive in this region (Sarasola et al. 2006). Post dispersal seed predation for pines in this system is largely carried by the native rodents *Oligoryzomys longicaudatus* and *Eligmodontia morgani* (Chiuffo et al. 2018). There is no evidence of secondary seed dispersal of *P. ponderosa* by animals in the study region (Nuñez et al. 2008, Chiuffo et al. 2018). On the study site, mean annual rainfall is 580 mm, concentrated during fall and winter (March-September), and mean annual temperature is 8.6 °C (San Ramon ranch meteorological station). Vegetation cover is ca. 60% being *Pappostipa speciosa* and *Festuca pallescens* the dominant plant species (Anchorena and Cingolani 2002).

Propagule pressure vs. seed predation

To evaluate the effects of propagule pressure and seed predation on seed survival, we conducted a seed predation experiment. We placed 18 transects every 50 meters perpendicular to a *P. ponderosa* plantation. In each transect, we installed plots at 0, 25, 50, 75, 100, 150 and 200 m from the plantation. To disentangle the effects of distance

from plantation and seed availability, we used two different experimental designs with different seed densities (variable and fixed). In the "Variable density" experiment we simulated natural seed dispersal on nine transects. The purpose of the "Variable density" experiment is to evaluate the proportion of seed rain that is consumed by seed predators at different distances from the pine plantation. Since data on seed production and dispersion is not available in our study site, we obtained data from the literature (Barrett et al. 1979, Krannitz and Duralia 2004). Specifically, we used data on the percentage of seeds that reach different distances from the edge of a *P. ponderosa* plantation and built a regression model that best described the variation in seed dispersal with distance from plantation. To fit this dispersion kernel we used a negative exponential model because it is appropriate for describing seed dispersal by wind (Clark et al. 2005, Lustenhouwer et al. 2017). Based on the dispersion kernel of this species we placed 100, 50, 30, 16, 10, 6 and 2 seeds per m² at 0, 25, 50, 75, 100, 150 and 200 m from the plantation respectively (Fig. 1). In the "Fixed density" experiment we put 20 seeds per m² at 0, 25, 50, 75, 100, 150 and 200 m from the plantation at each of the nine transects. We chose to put 20 seeds per m² because it represents an intermediate value between the mean (ca. 30 seeds/m²) and the median (16 seeds/m²) of the seed rain across all the distance levels. The purpose of the "Fixed density" experiment is to evaluate the effect of the distance from the pine plantation on seed predation. We glued the seeds to popsicle sticks with a non-toxic odor-free adhesive and we fastened sticks to the ground with stakes to prevent the removal of the sticks by seed predators. We used latex disposable gloves to handle the seeds to avoid imparting human odor to the seeds. We considered seed removal as evidence of seed predation (Nuñez et al. 2008, Chiuffo et al. 2018). In total, we placed 2013 seeds and 1473 sticks. We carried out this study during autumn because it is the period when pine seeds are naturally available (Krugman and Jenkinson 2008) and when rodent abundance tends to peak (Guthmann et al. 1997). We evaluated seed removal 30 days after installing the experiment.

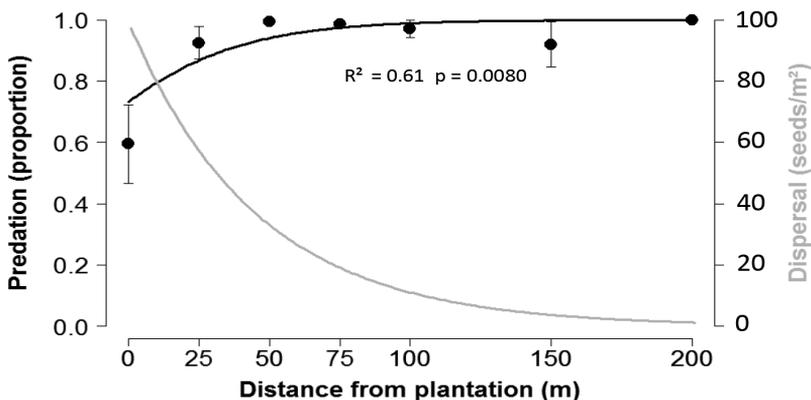


Figure 1. Seed dispersal (grey solid line) and seed predation (black dots and black solid line) of *P. ponderosa* at increasing distance from plantation. Dots show mean (+ SE) values of seed predation for each distance.

Missed mutualism

To test if lack of ectomycorrhizal fungi is limiting *P. ponderosa* invasion, we conducted a growth chamber bioassay. We collected ~100 cm³ of soil from each of the seven distances (0, 25, 50, 75, 100, 150, and 200 m) from the plantation on each of the nine "Variable density" transects used for the field experiment. Soils were collected during late fall 2017, just after the peak of mushroom fruiting season. Soil samples were dug from each site using an ethanol-sterilized spoon, placed in coin envelopes, each of which was then placed into gallon bags and then stored at 4 °C to be used the next day after collection. We removed small rocks and coarse roots and we used these soils to inoculate a mix of sterilized 50:50 soil and sand that had been autoclaved twice (Wolf and Skipper 1994). We randomly distributed soils from different distances into pots of 12 cm depth in ethanol sterilized plastic trays. To detect inadvertent EMF inoculation in the growth chamber, we randomly located 12 pots filled only with sterile soil. We used a total of 75 pots (nine for each from seven distances and 12 sterile) randomly distributed in five planting trays that were rotated once a week inside the growth chamber to avoid location effects. In each pot we planted four *P. ponderosa* seeds. We bought *P. ponderosa* seeds in the National Institute of Agricultural Technology (INTA – Bolson Experimental Station). We performed a pre-germination treatment in cold water for 48 hours. Floating seeds (vain seeds) were discarded and the rest were stored at 2–5 °C for three weeks. Prior to being sown, we surface-sterilized seeds in a 1% sodium hypochlorite solution. During the experiment water was added *ad libitum*, and there were no nutrients added to the pots. Light and temperature were kept constant. We used a cycle of 12 hours of light and 12 hours dark. Temperature was 25 °C during light periods and 10 °C during dark periods. Only the first plant to emerge was left in the pot, the rest were cut at the soil level avoiding soil disturbance. During the growing period we recorded survival (number of seedlings alive/total seedlings) for each distance and for sterile controls. After six months of initial planting we harvested the plants. We measured shoot height. We carefully rinsed clean seedling of adhering soil, separated them at the soil line into a root and shoot compartment, and placed them into an envelope to be dried in an oven at 65 °C for 2 days. We measured the biomass of dried root and shoot fractions separately using an electronic balance with accuracy to 0.0001 g. Prior to drying, we carefully examined the root system of each *P. ponderosa* seedling under a dissection microscope to address the extent of ectomycorrhizal colonization (based on morphological characteristics). Roots were placed on a petri dish, and the number of fine root tips colonized and not colonized by ectomycorrhizal fungi recorded.

Statistical analysis

To evaluate the effect of different predictive variables on seed predation we used logistic regressions. We calculated seed predation, the response variable, as the proportion of seeds that had been consumed (Orrock et al. 2015). To determine if distance from plantation and transect type had a significant effect on seed predation we considered them

as fixed factors. Because our experimental design included experimental units nested in transects we used transect number as a random factor (Bolker et al. 2009). To evaluate if the relationship between seed predation and distance from plantation differed between fixed and variable density transects we included the interaction between variables "distance" and "transect type" in our models. To compare seed predation between different distance levels we built logistic models with seed predation as response variable and distance level as a fixed categorical variable. Then we compared seed predation of each distance level with all the rest distance levels. To compare propagule pressure with seed predation at each distance level we also built logistic models with distance level as a fixed categorical variable. In this case we included a "dummy" distance level with 100% seed predation (proportion equal to 1) to represent propagule pressure. Then we compared seed predation of this "dummy" distance level with seed predation at distance levels 0, 25, 50, 75, 100, 150 and 200 m from the pine plantation. Significant differences would indicate that propagule pressure is higher than seed predation, while no significant differences would indicate that seed predation was as high as propagule pressure. For all our models we assumed a binomial distribution, using Generalized Linear Mixed Models (GLMM) based on Laplace approximation and a logit link function (lme4 package, glmer function) (Bates et al. 2015). We used analysis of deviance to evaluate the amount of total variation explained by each of the fixed factors (pseudo R^2).

To evaluate the effect of mycorrhizal inocula at increasing distances from plantation, we analyzed each response variable (survival, shoot height, biomass, and root colonization) separately. For response variables with binomial distribution (survival, and root colonization) we used GLMM fit by maximum likelihood and a logit link function (lme4 package, glmer function) (Bates et al. 2015). For those variables with a binomial distribution that presented overdispersion, we included an observation-level random effect for modeling the overdispersion (Harrison 2014). For response variables with normal distribution (shoot height and biomass) we used linear mixed-effects model fit by residual maximum likelihood (REML) (nlme package, lme function) (Pinheiro et al. 2017). In all cases we included "distance" as a fixed factor and "transect" as a random factor in the model. To address the effect of root colonization in plant growth we compared aboveground biomass (shoot dry biomass) of colonized vs. uncolonized seedlings with ANOVA (at $\alpha = 0.05$). All analyses were performed with R 3.4.0 statistical software (R Core Team 2018).

Results

Propagule pressure vs. seed predation

We found that seed predation increased with distance from plantation ($p = 0.0080$, Fig. 1, Table 1, 2). Propagule pressure was higher than seed predation only at the plantation edge (0 m) and seed predation surpassed propagule pressure at distances of 25 m and further from the plantation (Table 3). Seed predation was between 30 and 40% lower at the plantation edge than at distances of 25 m and further from the plantation (Fig. 1).

Table 1. Parameters of the logistic regression for seed predation of *P. ponderosa* at increasing distance from the pine plantation.

Fixed effects of distance from plantation, transect type and the interaction between distance and transect type.				
Significant effects ($p < 0.05$) are shown in bold letters				
Fixed effects	Estimate	Standard error	z	p
Distance	0.036	0.0134	2.656	0.0080
Transect type	-0.849	0.7791	1.090	0.2756
Distance * Transect type	-0.015	0.0203	0.731	0.4648
Random effects of the transect (n = 9). Intercepts are averaged				
Random effect	Mean intercept		Standard deviation	
Transect	1.475		0.000	

Table 2. Pairwise comparisons of seed predation levels between different distances from the pine plantation. Significant differences ($p < 0.05$) are shown in bold letters.

Pairwise comparison [m]	p value
0 vs 25	0.0396
0 vs 50	0.0397
0 vs 75	0.0312
0 vs 100	0.0291
0 vs 150	0.0441
0 vs 200	0.0438
25 vs 50	0.4273
25 vs 75	0.5187
25 vs 100	0.6585
25 vs 150	0.9311
25 vs 200	0.4090
50 vs 75	0.8348
50 vs 100	0.6705
50 vs 150	0.3914
50 vs 200	0.9541
75 vs 100	0.8175
75 vs 150	0.4724
75 vs 200	0.7925
100 vs 150	0.6011
100 vs 200	0.6359
150 vs 200	0.3758

Table 3. Pairwise comparisons between propagule pressure and seed predation for each distance level from the pine plantation. Significant differences ($p < 0.05$) are shown in bold letters.

Pairwise comparison	Distance [m]	p value
Propagule pressure vs seed predation	0	0.0438
	25	0.4090
	50	0.9541
	75	0.7925
	100	0.6359
	150	0.3758
	200	1.0000

Moreover, we found no effect of transect type (variable vs. fixed) on seed predation ($p = 0.2756$). The relationship between seed predation and distance from plantation was the same for both transect types as evidenced by the absence of interaction between distance from the plantation and transect type ($p = 0.4648$). With the fixed density transects we

offered 25%, 100%, 230% and 900% more seeds than would naturally be available at 75, 100, 150 and 200 m from the plantation, respectively, but still seed predators consumed 97%, 98%, 88% and 100% of the seeds dispersed, respectively.

Missed mutualism

EMF root colonization decreased with distance from plantation ($p = 0.0139$, Fig. 2, Table 4). Mean EMF root colonization at the pine plantation edge was ca. 90% while it decreased to ca. 40% at the furthest distance from the plantation evaluated here (200 m). However, we found no differences in seedling growth (shoot height and biomass) or survival with distance from plantation ($p_{\text{height}} = 0.6387$; $p_{\text{biomass}} = 0.9911$; $p_{\text{survival}} = 0.4830$). When we pooled all distance levels together and compared seedlings colonized by EMF (ranging from 10% to 100% EMF root colonization) with seedlings un-colonized (0% EMF root colonization) we found differences in seedling biomass favoring colonized seedlings ($p = 0.0400$, Suppl. material 1: Fig. S1). Finally, the roots of the seedlings growing in sterile soils were not colonized by EMF, showing there was no inadvertent EMF inoculation in the growth chamber.

Table 4. Parameters of the logistic regression for EMF root colonization at increasing distance from the pine plantation.

Fixed effects of distance from plantation. Significant effects ($p < 0.05$) are shown in bold letters				
Fixed effects	Estimate	Standard error	z	P
Distance	-0.012	0.0050	-2.460	0.0139
Random effects of the transect (n = 9), and the observation included due to overdispersion. Intercepts are averaged				
Random effect	Mean intercept		Standard deviation	
Transect	2.064		0.396	
Observation	2.027		1.554	

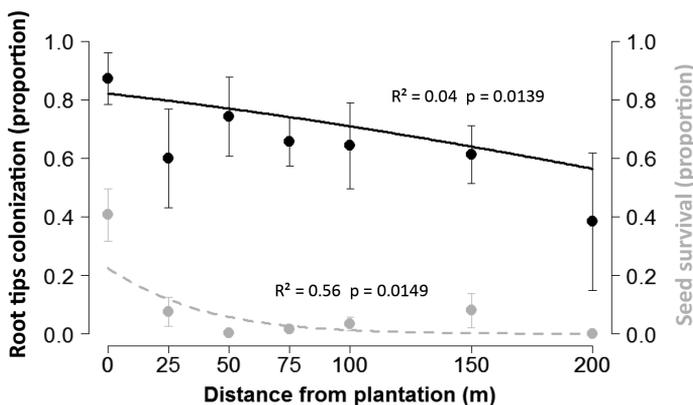


Figure 2. EMF root tips colonization on growth chamber bioassay (in black) and seed survival from predation for *P. ponderosa* on field experiment (in grey) at increasing distance from plantation. Dots show mean (+ SE) values for each distance.

Discussion

Our results provide strong empirical evidence that seed predation may be the most important biotic mechanism limiting *P. ponderosa* invasions in this system. Seed predators consumed ca. 95% of the seeds dispersed outside the pine plantation, thereby limiting seed availability. Propagule pressure varied in a wide range, from 100 seeds/m² at the plantation edge to 2 seeds/m² at a distance of 200 m from the plantation, however, only at the plantation edge does propagule pressure overwhelm seed predation. At distances of 25 m and further from the pine plantation, seed predation overcame the influence of propagule pressure. This is particularly clear when we consider seed predation in the fixed density transects: at distances from plantation higher than 75 m seed predation was between 25% and 900% higher than propagule pressure. This survival pattern of not predated seeds suggests that *P. ponderosa* seedling annual recruitment is limited to the first 25 m from the seed source. Altogether, these results provide evidence of how biotic resistance from generalist natural enemies can hinder an invasion.

In this study, we found that pine seed predation probability increases with increasing distance from plantation (Fig. 1). This fits with the seedling recruitment pattern described by McCanny (1985). Such inverse density-dependence seed mortality can be explained by predator satiation (Janzen 1971), when predators are satiated by the higher seed densities near adult plants (Augspurger and Kitajima 1992). Predator satiation near the pine plantation makes sense when we consider the gradient of decreasing seed availability with increasing distance from the plantation (Fig. 1). An alternative explanation is that native predators' activity is lower near pine plantations because it is a type of habitat very different from the steppe. The low cover of understory vegetation in pine plantations (Paritsis and Aizen 2008) means that small mammals (main pine seed predators in this region) (Nuñez et al. 2008, Chiuffo et al. 2018) have few sheltered habitats to take refuge from predators. Thus, small mammals would reduce risk by spending less time searching for food in the understory of pine plantations than in the adjacent low stature vegetation. The observed seed predation pattern may not reflect *P. ponderosa*'s probability of establishment and invasion because many other factors and processes are involved between seed survival and invasion success (Richardson et al. 2000, Carrillo-Gavilan et al. 2010, Blackburn et al. 2011). However, if seed predators are able to consume most of the seed production of a plant population then its probability of becoming invasive would be highly reduced (Pearson et al. 2012), especially considering further filters before invasion success (Richardson et al. 2000, Blackburn et al. 2011, Carrillo-Gavilan et al. 2012).

Our results also show that the abundance of EMF may not be limiting pine seedling survival and growth at the distance range evaluated. We found a gradient of decreasing root colonization with increasing distance from plantation (Fig. 2). However, this root colonization gradient did not affect seedling survival or growth. Other studies have found important effects of EMF abundance and composition on pine seedling establishment (Nuñez et al. 2009, Hayward et al. 2015, Urcelay et al. 2017) but working with wider distance ranges. For example, Nuñez et al. (2009) found effects of EMF on *P. ponderosa*

seedling establishment working with a distance of 1000 m from plantation. Lower levels of EMF root colonization at further distances from the pine plantation may have a significant effect on seedling growth. In this vein, we found that only when seedlings were not colonized (0% EMF root colonization) their growth was negatively affected (Suppl. material 1: Fig. S1). However, if seed survival to predation is limiting at a distance of 25 m from plantation (Fig. 2) the importance of limitations at further distances may not be fundamental to explain invasion in the first hundreds meters from plantations, but may be extremely important for rare but potentially key events of long distance dispersal. Another possibility is that the duration of the bioassay was not long enough to show the effect of root colonization on seedling growth. For example, Nuñez et al. (2009) found effects of *P. ponderosa* root colonization by EMF on seedling growth on a greenhouse experiment that lasted 9 months. However, on a growth chamber the effect of EMF on pine seedling growth should be clear after 6 months, as shown in other studies (Alberton and Kuyper 2009, Hazard et al. 2017). Therefore, our results suggest that EMF availability is not limiting *P. ponderosa* invasion in our study system but that the community of seed predators is behind the observed pattern of low invasion levels for *P. ponderosa*.

Our study shows that biotic resistance can be extremely important in plant invasions, even more important than the propagule pressure and the missed mutualism hypothesis. By contrast, a review of the empirical evidence for general hypothesis in invasion ecology found more support for the propagule pressure hypothesis than for the biotic resistance hypothesis in experiments with terrestrial plants (Jeschke 2014). This highlights the idiosyncratic nature of the importance of these hypotheses. In our study system, only the highest level of propagule pressure overwhelms biotic resistance, highly limiting the invasion of *P. ponderosa*. Therefore, our study highlights the role of biotic resistance in plant invasions.

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Supplementary material I

Figure S1.

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Data type: multimedia

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