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Repeated Stand-Replacing Crown Fires Affect Seed Morphology and Germination in Aleppo pine

Antonio Saracino[1]*, Alessandro Bellino[1][†], Emilia Allevato[1], Antonio Mingo[1], Stefano Conti[1], Sergio Rossi[2,3], Giuliano Bonanomi[1], Domenico Carputo[1] and Stefano Mazzoleni[1]

[1] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy, [2] Département des Sciences Fondamentales, Université du Québec à Chicoutimi, Chicoutimi, QC, Canada, [3] Key Laboratory of Vegetation Restoration and Management of Degraded Ecosystems, Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China

Post-fire reproductive niche of Aleppo pine (*Pinus halepensis*) is deeply interlaced with fire products. Indeed, the high pH and low osmotic potentials of ash beds under burnt crowns constitute the main constraints to seed germination. In this study, we aim to investigate whether fire recurrence, through the physico-chemical constraints imposed by the ash beds, affects the reproduction ability of *P. halepensis* at the germination stage. To this aim, Aleppo pine seeds were collected in neighboring even-aged stands subjected to 0, 1, or 2 fires (namely fire cohorts), and seed morphology and germination performance, in terms of cumulative germination and germination kinetics, were studied under increasing osmotic potentials (from 0.0 to −1.2 MPa) and pH (from 6 to 11). Besides fire history, the role of ontogenetic age of mother plants on seed morphology and germination was also investigated. Differences in seed morphology among the three cohorts have been highlighted in a multivariate context, with anisotropic enlargement of the seeds produced by pine stands experiencing repeated fires. The patterns of seed germination varied primarily in relation to the fire cohort, with seeds from the pine stand experiencing repeated fires exhibiting enhanced tolerance to pH stress. Conversely, germination performances under osmotic constraints mainly depends on tree ontogenetic stage, with an involvement of fire history especially in the timing of seed germination. Our results suggest that, at least in the short term, fire recurrence does not constrain the reproduction ability of Aleppo pine. These results highlight the need for further research to elucidate the mechanisms behind these responses to recurrent fires.

Keywords: fire recurrence, Mediterranean, seed germination, seed morphometry, osmotic potential, *Pinus halepensis*, pH, tree ontogeny

INTRODUCTION

Fire disturbance plays a crucial role in shaping plant communities and selecting plants with fire-related traits in Mediterranean ecosystems. Perennial, non-resprouting species with post-fire seeding ability (obligate seeders *sensu* Pausas and Keeley, 2014) are particularly sensitive to the length of fire return intervals, i.e., the time between two successive fires. These species are particularly affected by fires occurring during the immature stage of their life cycle, before seed reserves have built up, or during senescence, when they are already depleted (Zedler, 1995). Serotiny, the late release of ripe seeds from cones or fruits (Lamont et al., 1991), is a peculiar trait

allowing species to maintain a persistent hanging canopy seed bank, usually accessed in hot and dry conditions generated during fire events or by wind (Nathan et al., 1999; Rossi et al., 2017).

Aleppo pine (*Pinus halepensis* Miller) is a perennial species that grows mainly in fire-prone areas along the coast and in the interior of the Mediterranean Basin forming, at landscape level, variable patches of single-cohort stands which can locally disappear when fire is absent over a century (Agee, 1998). The species has a very short juvenile phase (Thanos and Daskalakou, 2000; Climent et al., 2008; Espelta et al., 2008), and builds canopy seed banks (Daskalakou and Thanos, 1996) consisting of both serotinous and non-serotinous cones with anatomically-different scales (Moya et al., 2008), whose ratio is dependent on both environmental and ontogenetic factors (Martín-Sanz et al., 2016). During its life cycle, this species progressively modifies the microenvironment to which its offspring are exposed through pre-fire litter production and necromass retention in the crown, which in turn affect fire intensity and post-fire products (Schwilk and Ackerly, 2001). The physical modification of the surroundings by pine trees is an example of niche construction by inceptive perturbation as labeled by Odling-Smee et al. (2003). The post-fire reproductive niche of Aleppo pine is spatially restricted under the crown projection and radially asymmetric for germination, seedling establishment and growth (Ne'eman, 2000). Ontogenetic modification of the crown shape, from conical to umbrella-like across the life cycle (Shmida et al., 2000) progressively increases the crown surface projection from 20 to 90 m² in trees without crown lateral competition in the old growth stage (Saracino et al., 2002). Low canopy base height (Mitsopoulos and Dimitrakopoulos, 2007, 2014), no self-pruning of dead branches (Keeley and Zedler, 1998; Schwilk and Ackerly, 2001) and no shedding of old open cones (Shmida et al., 2000; Saracino, personal observation) contribute to the propagation of fire from the forest floor up to the crown and an increase in the canopy fuel load. In addition, canopy bulk density actively supports crown fires (Mitsopoulos and Dimitrakopoulos, 2007, 2014). These traits represent a fire-embracing strategy (Schwilk and Ackerly, 2001; Pausas, 2015) aiming to promote the post-fire niche regeneration of Aleppo pine by producing a spatially heterogeneous thickness of ashes (Cerdà and Doerr, 2008).

The intensity of fire affects the quality and quantity of the ashes under the burnt canopy of Aleppo pine trees. Complete plant litter oxidation produces whitish-gray mineral ashes rich in soluble cations (Liodakis et al., 2005), whereas partial oxidation leaves organic components charred, dark and still rich in carbon (Bodí et al., 2014). The ash layer thickness decreases from the stem to the edge of the crown projection, following the radial asymmetry of pre-fire litter accumulation. Increasing ash thickness can inhibit seed germination (Paula et al., 2009) due to the increasing pH and reduction in osmotic potential (Henig-Sever et al., 1996). Moreover, repeated stand-replacing crown fires can reduce growth and reproductive fitness due to a delay in the onset of pine reproduction and a reduction in the number of reproductive pines and cone crop per tree (Eugenio et al., 2006; Espelta et al., 2008). By contrast, Ne'eman et al. (2004) and

Goubitz et al. (2004) documented higher levels of serotiny as a positive response to fire.

To sum up, post-fire germination depends on a number of environmental, genetic, and ontogenetic factors which can affect regeneration processes. These factors may be strongly interlaced and it is difficult to assess their role under natural conditions.

In this study we aim to investigate the effect of two main constraints exerted by post-fire substrate conditions, namely osmotic potential and pH, on seed germination both in relation to fire history and ontogenetic age of mother plants. To this end, germination behavior of seeds collected from Aleppo pine cohorts experiencing different fire histories were investigated under simulated conditions of post-fire substrates. Since morphology of the seeds could be involved in germination behavior, a set of morphological traits were also investigated in relation to ontogenetic stage and fire recurrence.

We selected stands located on costal sand dunes in Southern Italy, where the spatially heterogeneous recurrence of summer fires offered a unique opportunity to investigate the role of fire histories under homogeneous environmental conditions.

MATERIALS AND METHODS
Study Area and Seed Collection

The study area is located in a native Aleppo pine forest growing on coastal sand dunes in the Biogenetic Natural Reserve Stornara in Apulia region, Southern Italy (40° 27′ 43.46″ N, 16° 55′ 00.59″ E) (**Figure 1**). The soil is a loose siliceous-calcareous sand (Dumontet et al., 1996). Pine is associated with evergreen sclerophyllous (*Pistacia lentiscus*, *Phillyrea angustifolia*, *Myrtus communis*, *Rhamnus alaternus*, *Rosmarinus officinalis*), and malacophyllous (*Cistus salvifolius*) shrub species. The climate is semiarid Mediterranean, with a mean annual temperature of 15.8°C, and total precipitation of 535 mm.

Three patches with different fire histories were selected within the same forest, in order to minimize environmental variability and isolate the role of fire histories. Selection was done by using the fire register of the National Forest Service (since 1960) and fire maps drawn up by means of an aerial photogrammetric chronosequence (1947, 1954, 1974, 1990, 2002). In this forest, two stand-replacing crown fires occurred, partially overlapping and forming three patches with different fire history, burning 0, 1, and 2 times (FC0, FC1, and FC2, respectively, **Table 1**; **Figure 1**). In FC0, in addition to the analysis of aerial photos, the absence of recent fires was confirmed by the absence of charcoal fragments in the first 40 cm soil profile. In January 2008, 50–100 cones older than 2 years were collected from the whole perimeter of the middle and upper crown of 10 randomly selected plants from each fire cohort. Tree total height and diameter at breast height were measured, using a Tandem clinometer (Suunto, Finland) and a meter tape, respectively, to be used as a proxy of ontogenetic age, for all sampled trees (**Table 1**). In order to accurately represent the seed pool accessible during a fire event, both brown and serotinous cones were collected.

Cones from each plant were separately dried over 1 week in a well-ventilated and shaded environment, put in jute bags and moved to an oven ventilated at 50°C to facilitate scale shrinkage.
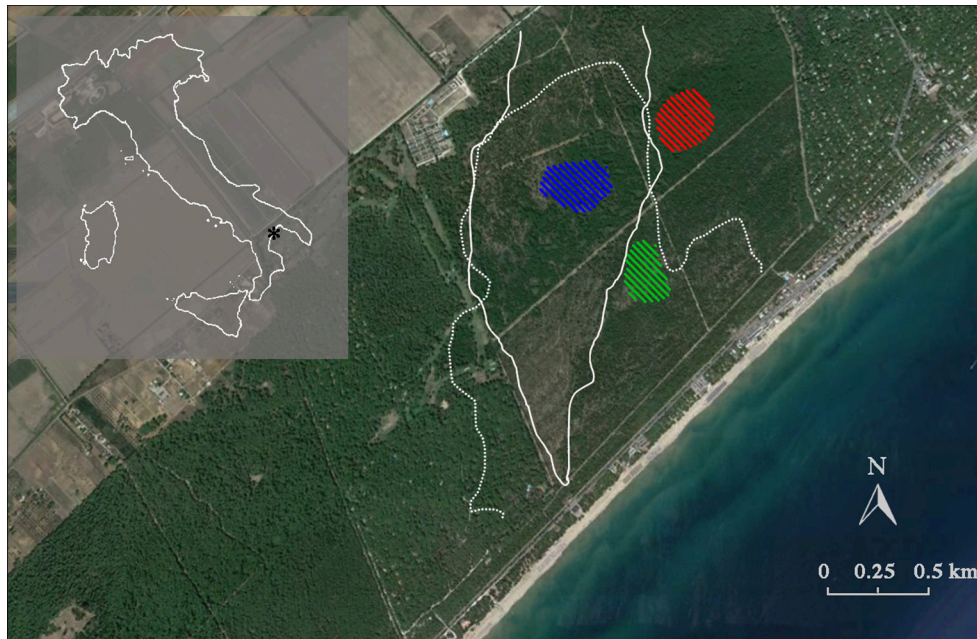
FIGURE 1 | Location of the study site, showing Italy (shaded area) with the relative position of the Biogenetic Natural Reserve Stornara in Apulia region (*). The native Aleppo pine (*Pinus halepensis* Miller) forest is registered in the National Book of Seed Forests managed by the Italian Forest Service (MAF, 1960) and is also recognized as a selected seed stand for Aleppo pine of the Mediterranean Basin due to its importance as a germplasm resource (Registration number 52–55, Castellaneta, FAO, 1997). White lines represent the stand-replacing crown fire boundaries (1970: dotted line; 1994: solid line). Diagonal line patterns indicate the tree sampling sites. Red: fire cohort 0 (FC0); green: fire cohort 1 (FC1); blue: fire cohort 2 (FC2).

TABLE 1 | Fires and trees characteristics of the three fires cohorts with different fire history, burning 0, 1, and 2 times (FC0, FC1, and FC2, respectively).

Fire cohorts	Fire dates	Tree characteristics		
		DBH (cm)	Height (m)	Age (years)
FC0	–	55.8 ± 10.1	16.4 ± 1.2	85
FC1	July 1970	24.5 ± 2.9	9.0 ± 0.7	37
FC2	July 1970 and August 1994	15.9 ± 4.0	8.4 ± 1.2	13

Values are given as mean ± s.d. (n = 10 for each fire cohort). DBH represent the diameter at breast height.
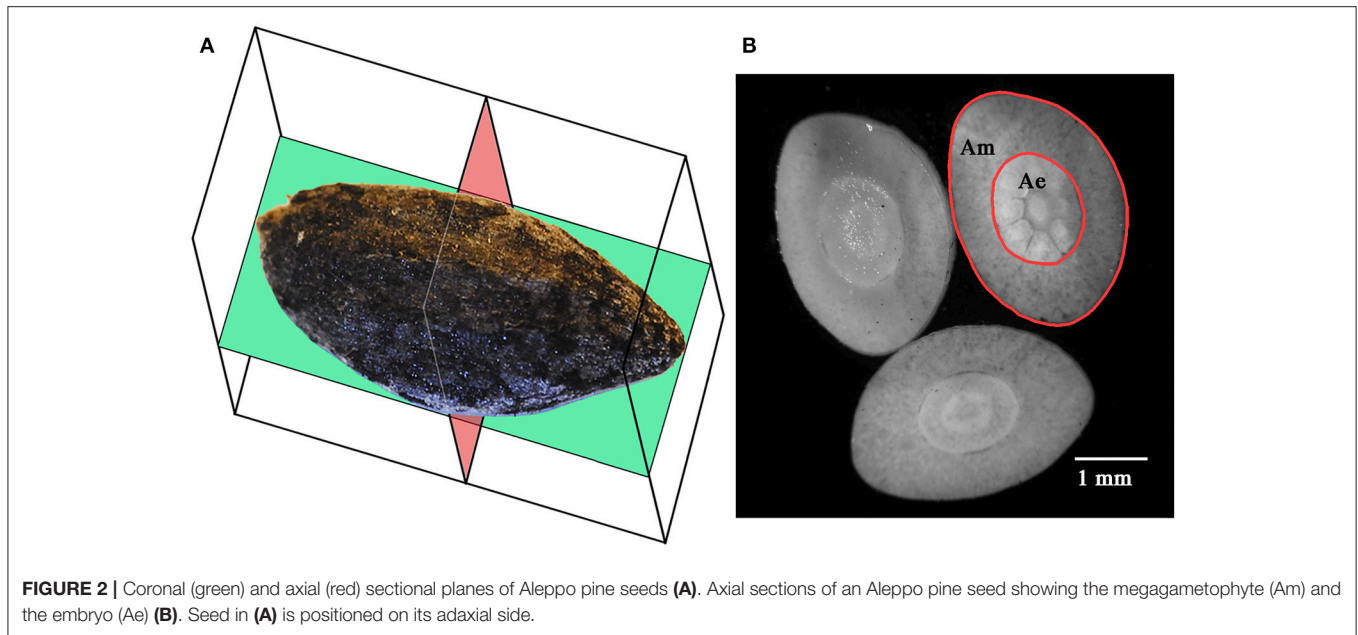
The seeds extracted were stored in vacuum-type PVC bags at 4°C. From each of the different fire cohorts at least 1200 seeds for the germination tests and 125–150 for morphometric analysis were used. Seeds from the various plants of each fire cohort were pooled together in the germination experiment, in order to investigate the overall germination responses of each cohort. The seeds were carefully selected from those with intact coats. A preliminary cutting test allowed the determination of 11 mg as the threshold mass for fully developed seeds to be used in the experiments. Unwinged seeds were weighed by means of a semi-micro balance to the nearest 10^{-2} mg.

Morphometric Analyses

Morphometric analyses were performed on 125–150 seeds per fire cohort (125 from FC0, 125 from FC1, and 150 from FC2), for a total of 400 seeds. Each seed was photographed in its coronal/abaxial plane (**Figure 2A**; see Kolotelo, 1997 and Terskikh et al., 2005) at 2.5× magnification using an optical microscope (Dialux 20, Leitz, Germany) equipped with a CoolSnap K4 camera (Photometrics, USA). The coronal shape of each seed was then numerically derived from the digital images of the seeds through an *ad hoc* computer script (unpublished data) developed for the R programming environment (R Core Team, 2014), based on the codes reported in Claude (2008). The script (i) imported the binary images of the seeds (threshold performed using the ImageJ 1.43u software package), (ii) derived the Cartesian coordinates of each pixel on the contour of the seed, (iii) calculated the centroid of the contour points, (iv) rotated the point set around the centroid, and (v) compressed the information by storing the length of 36 vectors, from the centroid to the contour points, spread by 10° from each other. The shape of the seeds was then defined by 36 variables, each one corresponding to the length of a radius.

The seeds were carefully uncoated from the micropylar end using hand cutters and weighed again. Uncoated seeds were free-hand cross-sectioned with a razor blade (**Figure 2B**), obtaining three to five thin sections, which were mounted on a glass slide and photographed with the aforementioned apparatus. The digital images were then analyzed with the free software package ImageJ 1.43u (http://rsb.info.nih.gov/ij/index.html), fitting their perimeters with polylines. The section with the largest surface was selected and used for the measurement

FIGURE 2 | Coronal (green) and axial (red) sectional planes of Aleppo pine seeds **(A)**. Axial sections of an Aleppo pine seed showing the megagametophyte (Am) and the embryo (Ae) **(B)**. Seed in **(A)** is positioned on its adaxial side.

of the section area of the embryo with and without the megagametophyte. The area of the megagametophyte and the mass of the coat were calculated as the difference between the previous measurements.

Germination Tests

Germination tests involved experiments under different osmotic potentials (Ψ_π) and pH. Seeds were germinated in 9 cm disposable Petri dishes over four layers of filter paper wetted with 10 ml of test solution. In order to minimize Ψ_π and pH variations over time, seeds were transferred to freshly prepared dishes every 3 days. Petri dishes were placed in the dark in a germination chamber set on a 13 h-15°C:11 h-25°C cycle. This experimental setting aimed to reproduce the environmental conditions experienced by seeds in the field during germination: seeds buried in the dark under 2–3 cm of black post-fire substrate (A. Saracino, unpublished results), at the night:day soil temperatures previously recorded during seedling emergence from October to November (Saracino et al., 1993).

In order to simulate the variable conditions of the post-fire field substrates (Bodí et al., 2014), the germination tests involved six Ψ_π levels between 0 and −1.2 MPa, and six pH levels between 6 and 11. Mannitol solutions were employed for the Ψ_π germination tests to avoid the toxic effect of polyethylene glycols on the germination of *P. halepensis* seeds (Leshem, 1966), whereas different buffered solutions were employed in the pH germination tests (pH 6.0: MES 50 mM; pH 7.0: MOPS 50 mM; pH 8.0 or pH 9.0: TRIS 50 mM; pH 10.0 or pH 11.0: CAPS 50 mM). Each treatment was replicated five times on batches of 20 seeds. Germinated seeds were counted daily and removed as soon as radicles had protruded until the 28th day after seeding. Ungerminated seeds were then tested for viability by means of tetrazolium tests to refer the germination percentage to the total of viable seeds.

Data Analysis

The overall differences among the fire cohorts in the morphological traits of the seeds were inferred through a multivariate analysis of variance (MANOVA). The mass of the uncoated seeds (Mu), the mass of the coat (Mc), the area of the megagametophyte (Am), the area of the embryo (Ae), and the area of the coronal plane (Ac), were included in the model as dependent variables. A generalized canonical discriminant analysis (CVA) was used to evaluate the contribution of the biometrical traits in differentiating the seeds among the three cohorts. Moreover, a MANOVA and a CVA were performed to analyse the variations in seed shape in relation to the number of fires, using the 36 radii defining the contour of the seeds as dependent variables and the fire cohort as the fixed factor. The relationship between each morphometric parameter and fire history was assessed through linear mixed models using the number of fires and the tree size (both height and diameter at breast height as descriptors of plant ontogenetic stage) as fixed factors and the mother plant as a random factor. The significance of fire history and tree size was then evaluated using a Kenward-Roger adjusted F-test.

Cumulative germination percentages (G_f) and mean germination time (MGT), defined as the time when half of the viable seeds had germinated, were selected as descriptors of seed germination performance. G_f values were logit-transformed, according to Warton and Hui (2011) using the equation:

$$G_f = (G_f + \varepsilon)/(1 - G_f + \varepsilon)$$

where $\varepsilon = 0.01$ to obtain a normal distribution.

The relation between G_f or MGT with fire history and tree age was evaluated through ridge regression using the number of fires, tree age and Ψ_π or pH as predictors. Tree age, rather than tree

size, has been used in analysing germination data, since in the experiments, seeds from various plants were pooled together.

The ridge regression technique was chosen in relation to its L2 regularization, avoiding the shrinkage to zero of the weakest predictors, and to its usefulness in dealing with non-orthogonal problems (Hastie et al., 2009). Moreover, the predictors were scaled to mean zero and unit variance to allow their importance ranking in predicting G_f and MGT. The penalty parameter (λ) for model selection was chosen using the method proposed by Cule and De Iorio (2012).

The relationship between G_f or MGT and Ψ_π or pH was then modeled separately for the three fire cohorts using linear models and the differences in the slopes were tested with z-tests. All analyses and graphics were performed with the stats, lme4, lmtest, nortest, candisc, pbkrtest, ridge, and vioplot packages in R 3.1.1 (R Core Team, 2014).

RESULTS

Seeds from the three fire cohorts were differentiated in a multivariate context based on their size and mass (Pillai's trace = 0.36, $P < 0.001$), with a clear separation of FC2 seeds from those of the other two cohorts, the confidence circle of which partially overlapped. Morphometric traits differentially contributed to separate the three fire cohorts according to the decreasing order of the vector length: Ac, Mu, Ae, Mc, and Am (**Figure 3A**). The number of fires was significant in predicting Ac ($P < 0.05$) and Mu ($P = 0.05$), whereas neither the number of fires nor the tree size were significant in predicting the other

morphometric traits (**Table 2**). The variation in Ac and, to a lesser extent, in Mu among the cohorts is highlighted also in **Figure 3B**, showing the distribution of each morphometric trait in the three fire cohorts through violin plots. Seed shape was significantly affected by the number of fires (Pillai's trace = 0.54, $P < 0.001$) (**Figure 4A**), with an anisotropic increase in the seed coronal section (**Figure 4B**). Ridge regression results are reported in **Table 3**. In the Ψ_π experiment, all predictors, namely number of fires, tree size and Ψ_π, were significant in predicting the mean germination times, with the absolute value of the scaled coefficients ($|\beta|$) decreasing in the order tree age > fire number > Ψ_π. Conversely, tree age had the highest $|\beta|$ in the cumulative germination model and lower values were associated to Ψ_π and then fire number, with P-values greater than the 5% confidence limit. Overall, FC1 showed better germination in the Ψ_π, with higher G_f and lower MGT than the other two fire cohorts (**Figures 5A,C**). In the pH experiment, the number of fires had the highest $|\beta|$ both in the cumulative germination and in the mean germination time models. In the former, tree age $|\beta|$ was associated with a P-value greater than the 5% confidence limit. Furthermore, the number of fires determined an increase in G_f with a change in the slope in relation to pH, and a monotonic decrease in MGT (**Figures 5B,D**). Differences in the slopes of the analyzed parameters were observed only in the pH experiment for cumulative germination. Specifically, the slope decreased at increasing fire passages from −0.063 in FC0 to −0.001 in FC2 (**Figure 5B**). The slopes were significantly ($P < 0.001$ and $P < 0.05$, respectively) different from 0.0 in FC0 and FC1, but not in FC2.

FIGURE 3 | (A) Canonical variate analysis on seed morphometric traits (Mu, mass of the uncoated seed; Mc, mass of the coat; Ae, area of the embryo; Am, area of the megagametophyte; Ac, area of the coronal section of the seed). Fire cohorts are coded in different colors (FC0: red; FC1: green; FC2: blue). Vectors indicate the strength and influence of specific morphometric traits on point location relative to canonical axis 1 and axis 2. Confidence circles (for $\alpha = 0.05$) are also shown, enclosing the true mean of each group at the 95% confidence level and highlighting significant differences among groups in case of no overlap. **(B)** Violin plots for seed morphometric traits: [Mu: **B1**; Mc: **B2**; Ae: **B3**; Am: **B4**; Ac: **B5**]. White circles, black rectangle and upper and lower lines for each violin represent the median, the interquartile range and the upper and lower whisker, respectively. Colored bands represent the kernel density plot.
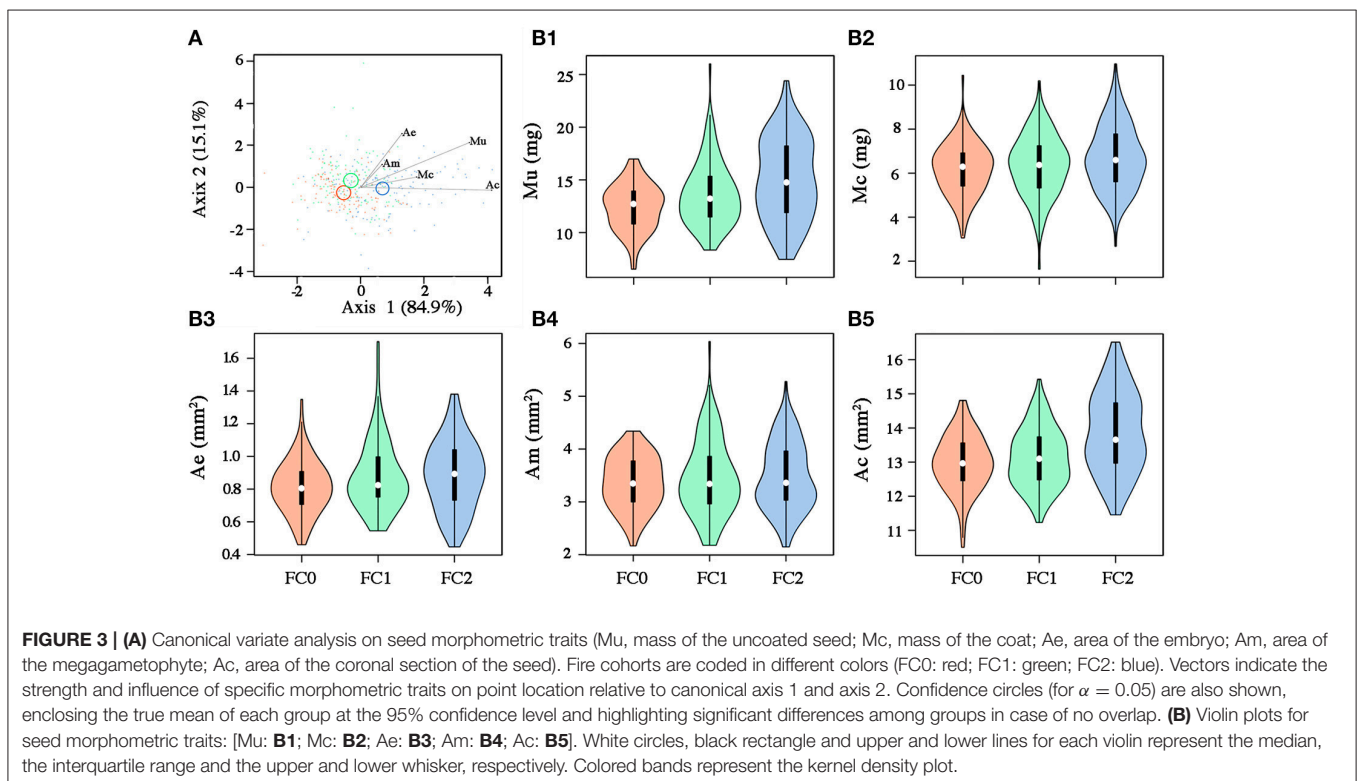
TABLE 2 | Summary of the linear mixed models for each morphometric trait (X: Ac, area of the coronal plane; Mu, mass of the uncoated seeds; Mc, mass of the coat; Am, area of the megagametophyte; Ae, area of the embryo) including fire number (left) and tree height (right) as predictors.

		Fire number						Tree height					
		Fixed effect		Random effect				Fixed effect		Random effect			
		Intercept	X	σ^2	$\tau_{00,P}$	ICC_P	R^2	Intercept	X	σ^2	$\tau_{00,P}$	ICC_P	R^2
Ac	B	12,875.67	456.91					14,065.04	−63.57				
	CI	12,400.57	104.07	6.3e+05	328,067.040	0.340	0.448	13,055.28	149.89	635,792.605	413,595.903	0.394	0.448
		13,350.76	809.75					15,074.80	22.75				
	P	<0.001	0.020					<0.001	N.S.				
Mu	B	12.35	1.32					16.14	−0.22				
	CI	10.69	0.09	5.755	4.101	0.416	0.499	12.78	−0.50	5.755	4.634	0.446	0.499
		4.01	2.55					19.50	0.07				
	P	<0.001	0.050					<0.001	N.S.				
Mc	B	6.14	0.27					6.90	−0.04				
	CI	5.48	0.22	1.179	0.623	0.346	0.381	5.64	−0.15	1.179	0.644	0.353	0.381
		6.79	0.75					8.17	0.06				
	P	<0.001	N.S.					<0.001	N.S.				
Am	B	4,194,885.83	91,088.68					4,411,950.47	−10,861.92				
	CI	3,816,414.51	189,999.32	3.48e+11	2.10e+11	0.377	0.405	3,683,511.80	−73,131.5	3.48e+11	2.14e+11	0.382	0.405
		45,73357.14	372,176.68					5,140,389.14	51,407.6				
	P	<0.001	N.S.					<0.001	N.S.				
Ae	B	822,495.60	35,547.96					942,648.35	−7,439.31				
	CI	718,117.20	41,974.54	2.24e+10	1.62e+10	0.419	0.453	743,061.89	−24,501.4	2.24e+10	1.62e+10	0.421	0.453
		926,874.00	113,070.47					1,142,234.80	9,622.8				
	P	<0.001	N.S.					<0.001	N.S.				

B, coefficient value; CI, 95% confidence interval (upper and lower values represent the lower and upper limit, respectively); P, P-value; σ^2, within-group (residual) variance; $\tau_{00,P}$, between-group-variance; ICC_p, intraclass correlation coefficient; R^2, coefficient of determination.

FIGURE 4 | Canonical variate analysis **(A)** on seed coronal shape using the 36 radii length data, with confidence circles (for $\alpha = 0.05$) highlighted. Superimposition **(B)** of the contour of each seed from the three fire cohorts and of the vectors relative to the first canonical axis from **(A)**. The mean contour for each fire cohort is highlighted with thicker lines. In both **(A,B)**, fire cohorts are coded in different colors (FC0: red; FC1: green; FC2: blue).
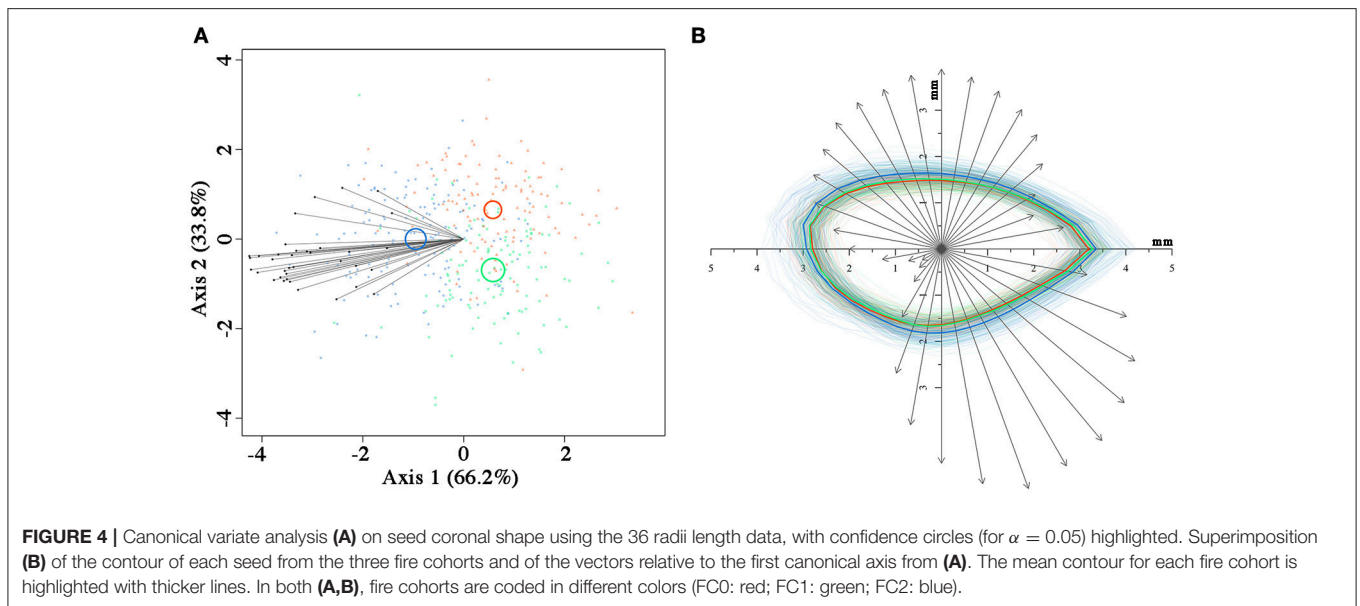
TABLE 3 | Summary of the ridge regression models for cumulative germination percentage (Gf) and mean germination time (MGT) in relation to osmotic potential (Ψ_π, left) and pH (right) as well as fire number and tree age.

		Intercept	Ψ_π	Fire number	Tree age	Intercept	pH	Fire number	Tree age
G_f	β_{ns}	2.432639	−0.056	0.020	−0.004	2.159004	−0.122	0.269	−0.007
	β		−0.208	0.158	−1.140		−2.308	2.491	−2.402
	s.e.m.		0.629	0.424	0.424		1.054	0.900	0.900
	t		0.331	0.373	2.691		2.189	2.766	2.668
	P		N.S.	N.S.	<0.01		<0.05	<0.01	<0.01
	λ		0.7559479				0.1389254		
MGT	β_{ns}	5.96820	3.228	2.699	0.088	16.34290	0.595	−1.248	0.013
	β		12.078	20.905	24.991		11.214	−11.565	4.274
	s.e.m.		0.974	2.457	2.457		3.178	3.544	3.544
	t		12.401	8.509	10.172		3.529	3.263	1.206
	P		<0.001	<0.001	<0.001		<0.001	<0.01	N.S.
	λ		0.02121095				0.08340478		

β_{ns}, non-scaled coefficient value; β, scaled coefficient value; s.e.m., standard error of β; t, t-value associated to β; P, P-value; λ, penalty parameter.
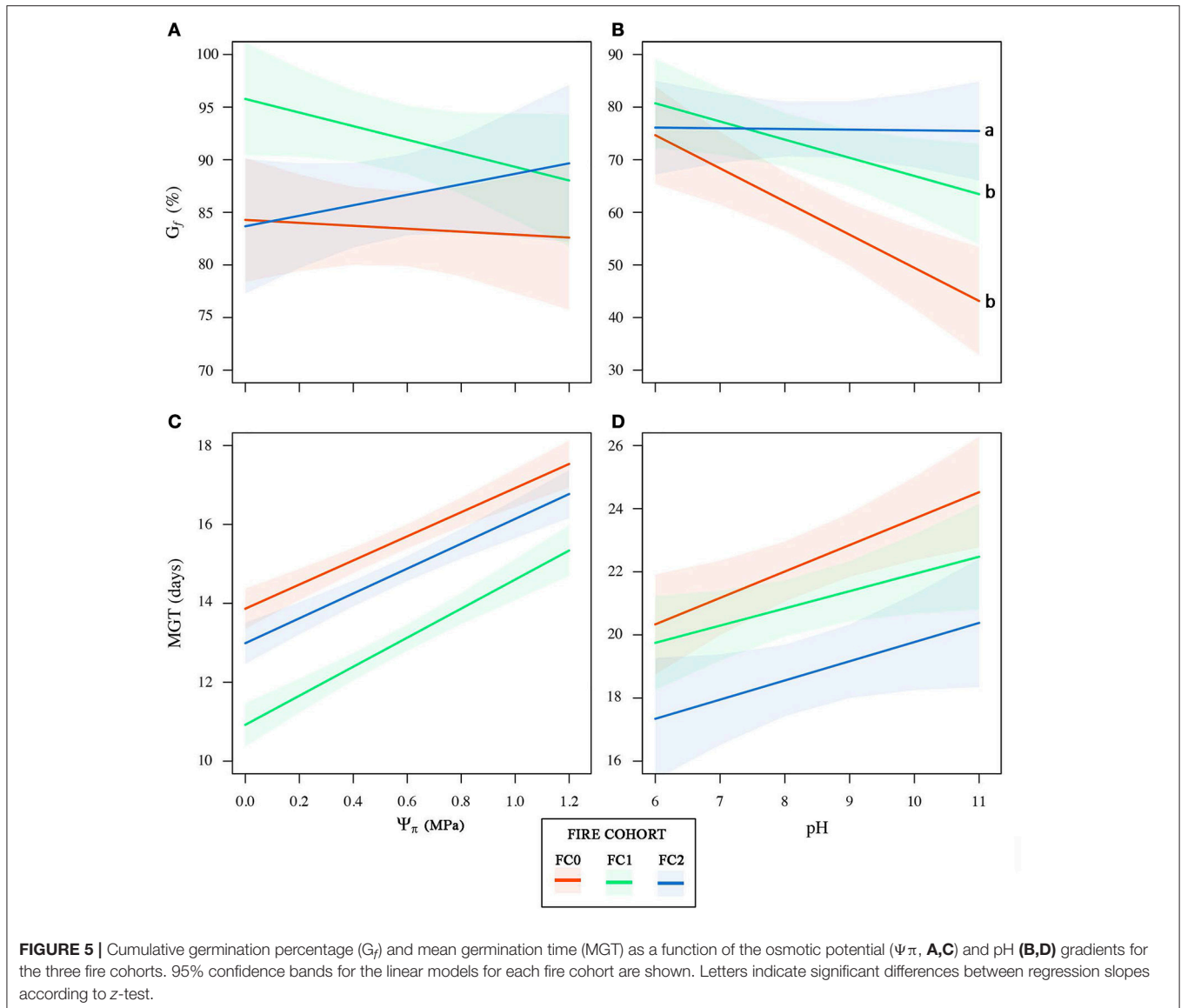
DISCUSSION

Seed Morphology

Differences in seed morphology among the three cohorts were highlighted in a multivariate context, with coronal area, the mass of uncoated seed and embryo size as the morphometric traits that contributed more to separate the three fire cohorts. The differentiation among cohorts is primarily related to the coronal area and the mass of the uncoated seeds, indicating an effect of the number of fires on these traits. Although variations in seed coronal area in relation to age/ontogenetic stage of the mother plants could not be definitively excluded, our result strongly suggests that the increase in coronal area, and to a lesser extent in other morphometric traits, is related to the fire history rather than to tree age. Indeed, young pine trees should produce fewer and smaller seeds than older trees in the same environment (Thanos and Daskalakou, 2000), whereas the opposite was observed in our study, supporting the hypothesis that seed size and shape are traits positively affected by fire. Our results could not confirm the influence of fire passages on the size of the megagametophyte and seed coat, which are considered important in the fire ecology of this species (Salvatore et al., 2010). Conversely, embryo size contributed to separate the three fire cohorts, and slightly increased from FC0 to FC2. Such an occurrence can be explained considering the importance of seed germination kinetics on seedling survival, which is a function of the embryo rather than of the megagametophyte. Indeed, larger embryos can germinate earlier (Vivrette, 1995) and overcome intraspecific competition for space, rather than nutrients, which are usually not limiting in post-fire substrates. Improved competitive advantage and seedling survival in larger Aleppo pine seeds, attributed to the higher number of cotyledons, were also reported by Daskalakou and Thanos (2010). Crown ontogeny may instead play a role in determining the higher variability in seed morphological traits exhibited by the younger fire cohort, since we collected cones on both stems and branches.

The variation in seed traits is often involved in plant evolution: in obligate seeders, seed-related traits like shape and mass (Weiher et al., 1999) usually have a major role in both post-fire seedling distribution and establishment (Hammill et al., 1998; Gómez-González et al., 2011, 2016; Shryock et al., 2014; Ruprecht et al., 2015; Jiménez-Alfaro et al., 2016). In our case the observed anisotropic increase in seed size, which leads to a change in seed shape, might affect a number of processes like dispersion, penetration and persistence in post-fire substrates, in which also seed wing traits may be involved. However, we cannot definitively exclude a non-adaptive age dependent plasticity in these traits. Therefore, this topic deserves further investigation.

Germination Patterns

In our study, the patterns of seed germination varied in relation to fire history, with seeds from FC2 exhibiting a greater ability to cope with the constraints imposed by the ash bed in the elective post-fire germination microsite of the crown projection area. Specifically, the number of fires improved seed tolerance to alkaline pH more than to the osmotic potential, both in terms of germination percentage and mean germination time. By contrast, the age of mother plants seems to have a greater effect on the germination performances under osmotic constraints. Cumulative germination showed the clearest responses, gradually shifting from marked inhibition at alkaline pH in FC0 seeds to a neutral response in FC2 seeds. Wood ashes of coniferous species typically reach pH values up to 11 (Raison, 1979; Thomas and Wein, 1990; Henig-Sever et al., 1996), which can inhibit germination. Indeed, Henig-Sever et al. (1996) considered the high soil pH induced by post-fire ash accumulation as the main abiotic factor controlling Aleppo pine forest regeneration, despite the promoting effect of nitrate and ammonium availability on seed germination (Henig-Sever et al., 2000). In addition, leaching by rain is usually not sufficient to produce a favorable seedbed before the occurrence of seed fall, as demonstrated in *P. banksiana* stands (Thomas

FIGURE 5 | Cumulative germination percentage (G_f) and mean germination time (MGT) as a function of the osmotic potential (Ψ_π, **A,C**) and pH **(B,D)** gradients for the three fire cohorts. 95% confidence bands for the linear models for each fire cohort are shown. Letters indicate significant differences between regression slopes according to *z*-test.

and Wein, 1994). The neutral response to pH of FC2 seeds suggessts that, at least in the short term, fire recurrence does not constrain the reproduction ability. Actually, it seems that after two repeated stand-replacing crown fires, Aleppo pine has the same, or even a better, capability to overcome the main abiotic constraint for the germination of seeds in post-fire substrates. Total germination under the osmotic constraint is above 80% in all fire cohorts, and age is the most important trait in predicting this behavior. This evidence suggests that fire frequency affects reproductive performance to a lesser extent than osmotic stress and is consistent with previous work which considers this environmental constraint more related to drought than to fire regimes (Falusi et al., 1983; Thanos and Skordilis, 1987). As such, in our case the high resistance to osmotic stress could be considered an adaptation to the semi-arid climate of the area. However, the potential interaction between drought and osmotic

potential deserves further investigation, since Aleppo pine seed germination occurs in autumn-winter, when soil is rewetted by precipitation.

The mean germination time pattern in relation to the osmotic potential was non-linear in relation to fire history, with FC1 seeds consistently germinating earlier than the others. This pattern may be related to the length of fire intervals (about 50 y for FC1 vs. 24 y for FC2). Indeed, mother plants of FC1 seeds were recruited on ash beds deeper and richer in soluble cations (Alifragis et al., 2001) than those of FC2 seeds, due to the higher litter and biomass accumulation during the FC1 life span. These features increase both fire intensity and the amount of ash deposited (Cerdà and Doerr, 2008; Bodí et al., 2014), suggesting an involvement of fire also in determining seed resistance to osmotic stresses.

Experimental results on the effects of the osmotic potential on Aleppo pine seed germination vary in relation to seed provenance (Thanos and Daskalakou, 2000). Henig-Sever et al. (1996) pointed out that osmotic potential only marginally affects the germination performance, and Thanos and Skordilis (1987) reported that Aleppo pine can show remarkable germination rates under osmotic stress attaining $\Psi_\pi > -1.46$ MPa. Conversely, Falusi et al. (1983) showed that Ψ_π ranging from -0.2 to -0.6 MPa can reduce germination rates and root growth in some provenances. However, literature results do not resolve the concomitant fire history and seed provenance effects, which limit the reach of the comparisons with our findings. It is well documented that repeated stand-replacing fires reduce the vegetative and reproductive fitness of Aleppo pine by delaying the onset of cone production, reducing the cone crop per tree and the number of reproductive pines (Eugenio et al., 2006; Espelta et al., 2008). In addition, future scenarios in the Mediterranean Basin predict an increase in fire frequency (Mouillot et al., 2002; Pausas and Keeley, 2009) due to a reduction in rainfall (IPCC, 2013). According to our findings, it seems that, at least in the germination phase, fire recurrence does not constrain the reproduction ability of *P. halepensis* due to production of seeds with increased tolerance to the constraints imposed by the ash bed. Instead, the neutral response in relation to pH entails the absence of ash pH constraint on the reproductive niche of the Aleppo pine cohort experiencing repeated fires, ensuring maintenance of fitness and population persistence in fire-prone areas. These changes should occur under several mechanisms implying genetic selection, at the germination stage, of seeds with better germination performance on ash beds. Under the genetic selection hypothesis, the ash layer selective pressure on seed germination would drive population adaptation to repeated fires, with future plants differentially recruited in relation to morphological, physiological or biochemical seed traits, and the eventual fixation of fire-related traits. As known, the environment can also provide adaptive transgenerational plasticity. This is often related to changes to seed provisioning and biochemistry as well as epigenetic mechanisms (reviewed by Herman and Sultan, 2011). They are environmentally sensitive and heritable, and thus play a key role in regulating transgenerational effects following plant stress conditions (Jablonka and Raz, 2009). In this perspective,

an alternative explanation of the differences observed among the fire cohorts could be related to short-term adaptation due to epigenetic modifications, e.g., post-translational changes of histone proteins, cytosine DNA methylation, action of small RNA (Danchin et al., 2011). These modifications are heritable, can all cause changes in gene expression and, as recently reported by Yakovlev et al. (2012), may represent an efficient mechanism to survive under changing environments. The age-dependent responses highlighted in this work can be considered positive traits to cope with frequent fire regime. Further studies are required to assess if they are phenotypic traits varying with growth and development, or if they are the result of long-term adaptation of this species to a fire prone environment.

We are confident that this work may open many research fronts, with further research involved in clarifying whether these observed short term responses are able to drive the local adaptation of new ecotypes, eventually allowing the maintenance of the reproduction ability of *P. halepensis* populations under recurrent fire regime.

AUTHOR CONTRIBUTIONS

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