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Direct and correlated responses to artificial selection for growth and water-use efficiency in a Mediterranean pine

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PREMISE: Persistence of tree populations in the face of global change relies on their capacity to respond to biotic and abiotic stressors through plastic or adaptive changes. Genetic adaptation will depend on the additive genetic variation within populations and the heritability of traits related to stress tolerance. Because traits can be genetically linked, selective pressure acting on one trait may lead to correlated responses in other traits.

METHODS: To test direct and correlated responses to selection for growth and drought tolerance in *Pinus halepensis*, we selected trees in a parental population for higher growth and greater water-use efficiency (WUE) and compared their offspring with the offspring of random trees from the parental population in two contrasting common gardens. We estimated direct responses to selection for growth and WUE and correlated responses for growth and tolerance to abiotic and biotic stressors.

RESULTS: We found a strong response to selection and high realized heritability for WUE, but no response to selection for growth. Correlated responses to selection in other life-history traits were not significant, except for concentration of some chemical defenses, which was greater in the offspring of mother trees selected for growth than in the offspring of unselected control trees.

CONCLUSIONS: The empirical evidence of direct responses to selection for high WUE suggests that *P. halepensis* has the potential to evolve in response to increasing drought stress. Contrary to expectations, the results are not conclusive of a potential negative impact of WUE and growth selection on other key life-history traits.

KEY WORDS   chemical defenses; correlated responses to selection; direct responses to selection; drought stress; global change; intrinsic water-use efficiency; Mediterranean pine; productivity; realized heritability; terpenes.

Global change is imposing biotic and abiotic threats on forests worldwide (Parmesan, 2006; Anderegg et al., 2015). In the Mediterranean basin, one of the areas most vulnerable to climate warming, it is predicted that drought stress and insect outbreaks will increasingly constrain forest productivity and persistence (Hódar et al., 2011; Ozturk et al., 2015).

How tree populations respond to these challenges will depend on their ability to adjust key functional traits related to stress tolerance. Changes in functional traits can be achieved either by phenotypic plasticity or long-term genetic adaptation (Nicotra et al., 2010; Anderson et al., 2012; Alberto et al., 2013). Phenotypic plasticity is the capability of an individual to alter its phenotype in response to a change in the environment (Nicotra et al., 2010). Plant plastic responses to drought involve a vast array of physiological adjustments, including stomatal regulation, osmotic adjustment,

reallocation of carbon reserves, and changes in allocation priorities to plant compartments (Ryan, 2011). Plants respond to biotic stress by producing new physical and chemical defenses when they perceive a biotic stimulus (induced defenses), enhancing plant resistance to the aggressor (Pieterse and Dicke, 2007). Although phenotypic plasticity is likely to mitigate some of the environmental impacts of rapid global change (Alberto et al., 2013; Matesanz and Valladares, 2013; Fox et al., 2019), adaptive changes may also occur, via genetic shifts in traits subject to selection (Aitken et al., 2008; Kremer et al., 2012). Genetic adaptation to biotic and abiotic pressures requires heritable intrapopulation variability in key functional traits related to stress tolerance (Bijma, 2011).

Heritability is defined as the degree to which a parental population is able to transfer heritable traits to its progeny (Bijma, 2011). Heritability of a given trait can be estimated in various ways (Walsh

and Lynch, 2018), but the most robust support of heritable change is empirical demonstration through selection experiments (Wray and Visscher, 2008). Realized heritability can be estimated in selection experiments by the ratio between the response to selection in the offspring population and the selection differential in the parental population (the breeder's equation; Walsh and Lynch, 2018); however, traits must be assessed for both generations, which is operationally difficult in forest trees due to their long generation cycles (Petit and Hampe, 2006).

Traits do not act independently. Beyond complex phenotypic interactions, traits can also be associated genetically due to linkage disequilibrium or pleiotropy (Walsh and Lynch, 2018). If traits are genetically related, selective pressure exerted on one trait may lead to correlated responses in others (Pujol et al., 2018). Therefore, selective pressures associated with global change (e.g., drought stress and insect outbreaks) may not only exert adaptive changes on traits directly related to stress (e.g., drought tolerance and plant defenses), but may also genetically alter other relevant life-history traits in subsequent generations (e.g., Santos del Blanco et al., 2015). Understanding how key adaptive traits are genetically integrated with each other is crucial for forecasting the long-term potential impact of global change on forest trees and other anthropogenic effects such as domestication and artificial selection for increased productivity. Although estimates of genetic correlations between traits are useful to infer correlated responses to selection (Walsh and Lynch, 2018), direct estimation through artificial selection experiments is, again, the best empirical way to explore the evolutionary consequences of genetic linkage among traits (Conner, 2003). Results from this approach are available for some timber-related traits (e.g., growth and wood quality) in the context of tree breeding programs (MacLachlan et al., 2017; Liziniewicz and Berlin, 2019), but are comparatively scarce for functional traits in less economically important species (Mátyás et al., 2009; Santos del Blanco et al., 2015).

We conducted a selection experiment in a Mediterranean pine species to estimate (1) direct responses to selection in key functional traits related to drought tolerance and tree growth rate and (2) correlated responses to selection in traits related to growth, plant chemical defenses, and drought tolerance. Individual trees of a parental Aleppo pine (*Pinus halepensis* Mill.; Pinaceae) population were phenotyped in a regular, monospecific stand. Trees with the highest growth or greatest intrinsic water-use efficiency (iWUE, i.e., the ratio of net photosynthesis to stomatal conductance to water vapor) were independently selected. Seeds from these trees were collected, and the resulting progeny, together with a control seedlot from unselected random trees from the same parental population, were established in two common gardens with contrasting environmental conditions. Direct and correlated responses to selection for iWUE and growth were estimated. We measured two important traits: one likely to predict tolerance to drought and the other related to resistance to biotic stress. These are two of the main stressors associated with global change (Anderegg et al., 2015). To estimate drought tolerance, we measured carbon isotope composition ($\delta^{13}C$) as a proxy of iWUE (Farquar et al., 1982). For isohydric species like Aleppo pine, fast stomatal closure in response to water deficit leads to ^{13}C-enrichment in plant tissues (Choury et al., 2017). To estimate resistance to biotic stress, we measured total mono-, sesqui- and diterpenes as proxies of investment in chemical defenses. Terpenes are the main constituents of pine oleoresin, the most important physical and chemical barrier in conifers to insect herbivores and

pathogens (Celedon and Bohlmann, 2019). Oleoresin is produced in large amounts in all tissues of pine trees and its synthesis requires abundant carbon resources (Gershenzon, 1994). Allocation of resources to resin-based defenses may therefore trade off with other vital functions such as growth, reproduction, and tolerance to abiotic stress (Agrawal et al., 2010). In addition, responses to water shortage and biotic stress may be related through cross-talk between the metabolic signaling pathways involved in these responses (Nguyen et al., 2016). Whenever trade-offs between different vital functions have a genetic basis, they may have important evolutionary consequences (Sampedro, 2014).

MATERIALS AND METHODS

Species studied

Aleppo pine (*Pinus halepensis* Mill.; Pinaceae) is a fast-growing, drought-avoiding Mediterranean conifer distributed mainly along the western Mediterranean coast. Across its natural range, the species shows substantial genetic variation, with pronounced genetic differentiation among populations in growth (Voltas et al., 2018) and $\delta^{13}C$-based iWUE (Voltas et al., 2008). Populations from drier environments have greater iWUE (i.e., higher $\delta^{13}C$) and lower growth rates than populations from wetter environments, probably because of adaptation to abiotic stress (Voltas et al., 2008).

Parental population and artificial selection

The parental population was a small, isolated Aleppo pine stand of ca. 2 ha, aged 13 ± 0.4 years (mean ± SE; $N = 130$), located in Cucalón (Castellón, Spain; 39°47′27.9″N, 0°37′01.1″W) and growing in a calcic cambisol under a Mediterranean climate. Average climate data for 1951–1999 was obtained from a regional climate model (Gonzalo, 2007). The climate is characterized by relatively low annual precipitation (513 mm) and pronounced summer droughts (82 mm accumulated rainfall from June to September). Mean annual temperature is 14.2°C, and annual thermal oscillation is 15.1°C.

In June 2014, 130 individual trees were randomly selected in the stand and characterized for growth (tree height and diameter at breast height), $\delta^{13}C$-based iWUE and chemical defenses. Increment cores were extracted at the base of all trees using 5-mm Pressler increment borers, as described by Santini et al. (2018). Wood core samples were oven dried at 60°C for 48 h, then sanded for tree-ring dating. Tree-rings corresponding to the years 2009–2013 were isolated and ground in a mixer mill (Retsch MM301, Haan, Germany) for iWUE estimation through carbon isotope composition ($\delta^{13}C$). Approximately 1 mg of the resulting powder was encapsulated and analyzed by GC-IRMS (see the extended method in "Chemical analysis" in the progeny trials section).

Bole resin flow and concentration of nonvolatile resin and total polyphenols in the needles and stems of terminal branches were used to characterize defensive investment of parental trees. Nonvolatile resin, composed mainly of diterpenes, is a good proxy of defensive allocation in pine trees (Sampedro et al., 2011), and bole resin flow is a good measure of resin exudation and defensive capabilities in adult trees (Lombardero et al., 2000). Bole resin flow was estimated by removing a disk of bark and phloem with an arch punch and measuring the resin flowing in the following 24 h (Appendix S1). Nonvolatile resin in needles and stems was extracted in hexane and estimated

gravimetrically according to Sampedro et al. (2011) (Appendix S2). Total polyphenols in needles and stems were extracted with methanol and determined colorimetrically using the Folin-Ciocalteu method according to Moreira et al. (2009) (Appendix S3).

After phenotyping the 130 parental trees, we selected the 20 trees with the highest diameter growth, the 20 with the greatest iWUE and the 20 with the highest reproductive investment, although reproductive investment was not included in the study. A random subsample of 40 of the 130 parental trees was used as a control group. Selected mother trees were interspersed within the stand, 3–30 m apart. In November 2014, we collected 2–3 mature pine cones from each tree in the groups selected for iWUE and growth and combined the seeds retrieved from the cones in each group to form the seedlots (control and selection groups). Additional seeds from the 40 control mother trees were collected at individual tree level, but the resulting open-pollinated half-sib families were not included in the study. Seeds were extracted by heating the cones at 60°C for 3–4 h and preserved at 4°C until sowing.

Progeny trials

Seeds were sown in March 2015 in a forest nursery in Ejea de los Caballeros (Zaragoza Province, Spain; 42°08′02.2″N, 1°12′35.4″W). Seeds from a local Aleppo pine stand in Ejea de los Caballeros were also included as a local control seedlot. This local seed source, formed by combining the open-pollinated seeds of a large number of mother trees, was included in the progeny trials to compare the performance of the control and selection groups and the local-origin trees.

Seeds were disinfected with copper oxychloride (Cuprebel 22; Kenogard, Madrid, Spain), then sown in 210-cm³ containers filled with a mixture of 70% peat and 30% local pine forest soil. Plants were grown outdoors in a shade house (20% shade) in ambient conditions with conventional nursery practices. Temperatures and air humidity during the growing period were −2 to 39°C and 36 to 100%, respectively.

In March 2016, when seedlings were 1 year old and 9.5 ± 0.1 cm tall (mean ± SE), two common gardens were established in contrasting climate and edaphic conditions: Montañana and Farasdués. The Montañana trial (Zaragoza Province, Spain; 41°42′50.6″N, 0°49′16.5″W) is on fertile alluvial agricultural land with a gleyic cambisol. Mean annual temperature and annual thermal oscillation are 14.9°C and 19.0°C, respectively. Mean annual precipitation is 350 mm and accumulated summer rainfall is 70 mm (June to September). Summer water stress was alleviated by flood irrigation every 15 days to total field capacity to further enhance conditions at this fertile site.

The second trial was in Farasdués, Ejea de los Caballeros (Zaragoza province, Spain; 42°13′ 25″N, 1°05′43 ″W), on stony land with a gleyic cambisol, near the Pre-Pyrenees mountains. Mean annual temperature and annual thermal oscillation are 14.0°C and 18.5°C, respectively. Mean annual precipitation is 448 mm, with a pronounced summer drought (94 mm accumulated from June to September). This site was not irrigated.

Average climate data for 1951–1999 was obtained for both sites from a regional climate model (Gonzalo, 2007).

Experimental design—The trials followed a randomized complete block design with eight blocks and six seedlots, including the four selection groups (control, iWUE, growth, and reproduction, although reproduction was not analyzed due to the young age of the seedlings) plus two replicates of the same local seed source. This basic design was augmented with the individual half-sib families of the control maternal trees, established following an α-lattice incomplete design superimposed onto the complete block design described above. Because we focused exclusively on comparing the selection groups, the underlying experimental design for the study was a randomized complete block design. All the selection groups and the local seed source were formed by combining the seeds from the corresponding mother trees (selected, control, or local), and therefore, no family structure was retained.

The experimental design comprised multi-tree plots, where each seedlot was represented in each block by six contiguous seedlings planted in rectangular plots (hereafter experimental units) of 3 × 2 plants at 3 × 3 m spacing. For the study, 480 seedlings (2 sites × 8 blocks × 5 seedlots [the three selection groups + 2 local seedlots] × 6 plant replicates per experimental unit) were considered.

Offspring assessment—In November 2017, when pines were 2.5 years old, we measured survival and total height of all seedlings. Selection for growth in the parental population was based on stem diameter, but due to the difficulty of measuring diameter in young pines and to avoid damage to plantlets, we measured tree height as growth trait in the offspring.

To assess iWUE and allocation to chemical defenses, we sampled 12 healthy needles from the southern-exposed middle part of the stem of each surviving seedling. We made composite samples from the 6 seedlings from each experimental unit (N = 80 composite samples, corresponding to 2 sites × 8 blocks × 5 seedlots). Approximately 300 mg (fresh mass) of needles was immediately extracted with 1000 μL GC-grade hexane (HiPersolv Chromanorm, ref. 83992.320; VWR International, Radnor, PA, USA), with dodecane (ref. 1.09658.0005; Merck KGaA, Darmstadt, Germany) and pentadecane (ref. 76510; Merck KGaA) as internal standards, in 4 mL glass vials for subsequent analysis of terpenes. Vials were vortexed, sonicated (20 min), and kept in darkness overnight, then the solvent was recovered, and the plant material was weighed after drying at 105°C.

The remaining needle material in each composite sample was immediately frozen (at −24°C) and then freeze dried to determine carbon isotope composition ($\delta^{13}C$).

Chemical analysis—Finely ground needle tissue (ca. 1 mg) was encapsulated in tin capsules (Sn 98 capsules, ref. 176.9809.26; Lüdi Swiss, Flawil, Switzerland), and the carbon stable isotope ratio was determined using a Finnigan MAT Delta C IRMS (Thermo Fisher Scientific, Waltham, MA, USA) at the University of Davis (USA). Carbon isotopic composition ($\delta^{13}C$) was calculated using the method of Farquar et al. (1982) with Vienna PeeDee Belemnite (VPDB) as the standard:

$$\delta^{13}C\ (\text{‰}) = \left[\frac{R_{sample}}{R_{std}} - 1 \right] \times 1000 \qquad (1)$$

where R_{sample} and R_{std} are the isotopic ratio $^{13}C/^{12}C$ in the sample and in the VPDB standard, respectively.

Concentration of mono-, sesqui-, and diterpenes was determined in the hexane extracts as a proxy of seedling allocation to chemical defenses. Analyses were performed as in López-Goldar et al. (2018). A 150-μL sample of the hexane extract was used to analyze mono- and sesquiterpenes by GC-flame

ionization detection (FID). For diterpene analysis, a second 150-μL sample of the hexane extract was dried under N_2, redilated in HPLC-gradient grade methanol (HiPerSolv Chromanorm, ref. 20864.320; VWR International) with heptadecanoic acid (ref. H3500; Merck KGaA) as internal standard, and methylated by adding tetramethylammonium hydroxide (1:10 v/v in methanol; ref. 334901; Merck KGaA). Terpenes were then separated and quantified by gas chromatography using a GC-FID Clarus 500 (Perkin Elmer, Waltham, MA, USA) equipped with an Elite-5 capillary column (30 m, ID 0.25 mm, film thickness 0.25 μm; Perkin Elmer) coupled to an FID and using the Total Chrom Navigator Clarus 500 v6.3.2 software (Perkin Elmer). The FID temperature was set at 300°C. The extract (1 μL) was injected into the GC port using splitless mode and hydrogen as the carrier gas. Instrument calibration and verification were performed using internal standards, alkane series (40 μg.mL⁻¹; Alkane Standard C8-C20, ref. 04070; Merck KGaA) and commercial standards (e.g., abietic acid 00010-25G, and dehydroabietic acid SMB00089-1MG; Merck KGaA; 40 μg.mL⁻¹). For mono- and sesquiterpenes, oven temperature was set at 40°C for 2 min, followed by a first temperature ramp of 4°C.min⁻¹ to 200°C, then a second ramp of 10°C.min⁻¹ to 250°C, and kept at this temperature for 5 min. For diterpenes, the oven was set at 152°C for 2 min, followed by a temperature ramp of 3°C.min⁻¹ to 260°C, and held at this temperature for 5 min.

Peaks were identified in the GC-FID by comparing the retention times and the Kovat index, calculated using a commercial alkane series with the retention times and Kovat index of the compounds identified in previous studies by GC-MS and GC-FID (López-Goldar et al., 2018; Suárez-Vidal et al., 2019). The minimum detectable peak area was set at 10,000 area units.

Statistical analyses

Frequency histograms were constructed for height and $\delta^{13}C$ data in the parental population using bin widths of 0.25‰ and 40 cm, respectively (the bin widths led to 10–12 bins and a good representation of the frequency distributions of the traits). Selection differentials (S = Mean of selected trees − Overall population mean) were calculated for $\delta^{13}C$-based iWUE and tree growth. Although selection for growth was based on diameter, we focused on height data, the trait assessed in the offspring. Because diameter and height in the parental population were highly correlated among each other ($R^2 = 0.79$, $N = 130$, $P < 0.001$), selection for diameter also included selection for height growth. Differences between control and selected groups (growth, iWUE) for the variables assessed in the parental population (height, diameter, $\delta^{13}C$, bole resin flow, nonvolatile resin, and total polyphenols in needles and stems of terminal branches) were analyzed by a one-way ANOVA.

Direct responses (height and $\delta^{13}C$-based iWUE) and correlated responses (survival, $\delta^{13}C$-based iWUE, height, and concentration of terpenes) to selection in the offspring were analyzed by fitting mixed models to the phenotypic data of the seedlots established in the two progeny trials. The mixed models assumed a complete block design within each trial, and included the seedlot (control vs. selected), the site (Montañana and Farasdués) and their interaction as fixed factors, and the block within site as a random factor. For seedling height, the only trait assessed at the individual tree level, the experimental unit (i.e., the interaction between seedlots and blocks within sites) was also included in the model as a random factor. Analyses were conducted separately for each selection trait

(growth vs. control, iWUE vs. control) using the MIXED procedure of SAS version 9.4 (SAS Institute, Cary, NC, USA; Littell et al., 2006). Plot–mean survival was arc-sine-transformed to achieve normality.

From these analyses, direct response to selection (R) for growth and iWUE was estimated as the difference of the least square means for the given trait between the offspring of the selected trees and the offspring of the unselected control trees. The traits were previously converted to a Gaussian N (0,1) distribution in the parental and the offspring populations to standardize effect size across the two generations. Realized heritability was then estimated as the ratio between the response to selection in the progeny and the selection differential in the parental population (both measured on standardized variables) assuming a coefficient of relationship of 1/3 ($h^2 = 1.5R/S$) (Walsh and Lynch, 2018). Seeds from the selection groups are from known selected mothers but unknown fathers. When mother trees are far apart (e.g., >30 m), they can be assumed to be unrelated (i.e., having different grandparents) and pollinated by different male parents (de Lucas et al., 2009). However, fine spatial structure at scales lower than 30 m could cause deviations from these assumptions. Although the probability of sharing the same male parent within open-pollinated families in anemophilous species like *P. halepensis* is low (Gaspar et al., 2009), it may be much higher in our study due to the small size of the parental population (a few hectares), the proximity between selected mothers (<30 m), and the high tree density in the stand. The theoretical coefficient of relationship of 1/4 for true half-sibs was therefore increased to 1/3 to account for this potential bias (Hernández-Serrano et al., 2014).

RESULTS

Selection within the parental population

The parental population averaged 4.4 ± 0.7 m in height and 7.0 ± 0.2 cm in diameter and presented a mean $\delta^{13}C$ of wood of −24.76 ± 0.05 ‰ (mean ± SE). Mean height of the parental trees selected for higher growth was 5.3 ± 0.9 m, a selection differential of 0.98 m (1.23 units of standard deviation for the standardized variable) (Fig. 1A). Parental trees selected for greater iWUE had a mean $\delta^{13}C$ of −23.91 ± 0.05‰, 0.85‰ (1.56 units of standard deviation for the standardized variable) higher than the overall mean parental population (Fig. 2A).

Artificial selection affected the phenotypic value only of the traits under selection (growth and $\delta^{13}C$), with no significant differences between the selected and the control groups for any other trait, including traits associated with defensive investment (Appendix S4).

Direct response to selection

Seedling growth and $\delta^{13}C$ differed significantly between the sites (Table 1). The Montañana seedlings were taller and less water-use efficient (i.e., lower $\delta^{13}C$) than the Farasdués seedlings (Fig. 3). Despite these site differences, response to selection for iWUE and growth was consistent across both sites (Fig. 3); i.e., no significant selection seedlot × site interaction was observed for either trait (Table 1).

No significant differences were found in early height growth between the offspring of growth-selected and unselected control trees (Fig. 1B; Table 1). Estimated realized heritability for height was therefore null (Fig. 1B). In the case of iWUE, the offspring of the trees selected for greater iWUE had higher $\delta^{13}C$ than in the
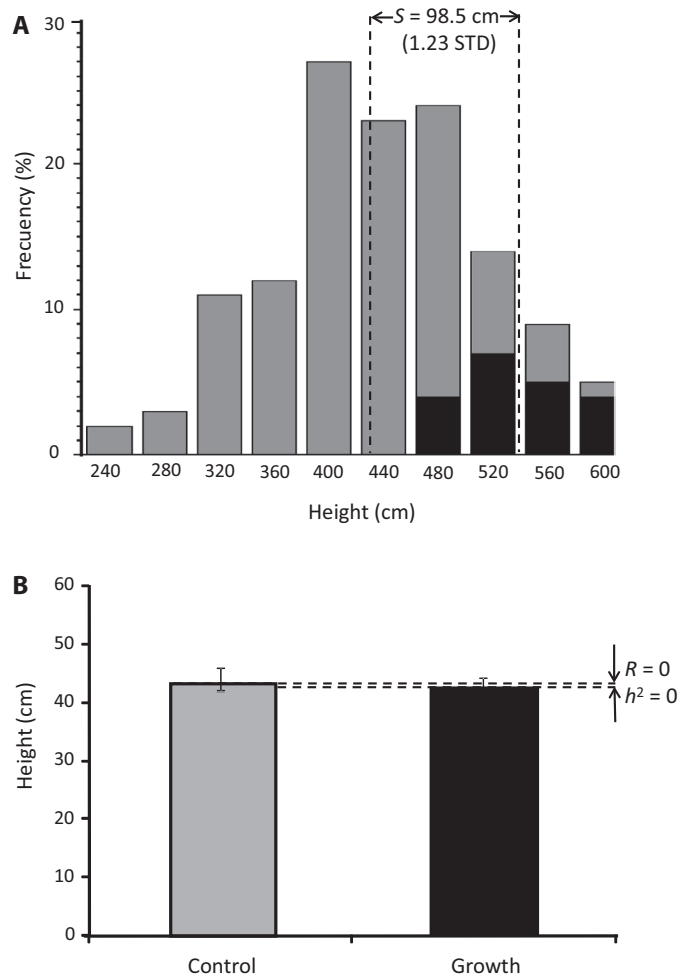
FIGURE 1. (A) Frequency histogram of tree height in the parental population (gray bars, N = 130) showing the selected trees with the largest growth (black bars, N = 20). Dashed vertical lines denote overall population mean and the mean of selected trees. The difference between the two values denotes the selection differential (S). Units of standard deviation (STD) for the standardized variable are in parenthesis. The differential selection in units of standard deviation is shown in brackets. (B) Average response to selection in the offspring population showing differences in growth (height, mean ± SE) between the offspring of growth-selected and control trees (N = 96 individual plants). Response to selection (R) and realized heritability ($h^2 = 1.5R/S$) are shown.

FIGURE 2. (A) Frequency histogram of $\delta^{13}C$ in wood of trees of parental population (gray bars, N = 130) showing the selected trees with the highest water-use efficiency (WUE) (black bars, N = 20). Vertical dashed lines denote overall population mean and the mean of selected trees. The difference between the two values denotes the selection differential (S). Units of standard deviation (SD) for the standardized variable are in parenthesis. The differential selection in units of standard deviation is shown in brackets. (B) Average response to selection in the offspring population showing differences in average WUE ($\delta^{13}C$ in needles, mean ± SE) between intrinsic WUE-selected trees and control trees (N = 16 composite samples). The difference between the two values denotes the response to selection (R). The response to selection in units of standard deviation is shown in brackets. Realized heritability ($h^2 = 1.5R/S$) was estimated on the response to selection and the selection differential obtained after standardizing the variable [N (0,1)] within each generation.

offspring of the unselected control group, regardless of the testing site (Fig. 2B; Table 1). Response to selection was 0.66‰ (0.90 units of standard deviation for the standardized variable), resulting in a realized heritability of 0.86.

Correlated responses to selection

Selection for growth or iWUE had no correlated effects in any of the traits analyzed in the offspring except for concentration of mono- and sesquiterpenes (Table 1), which were significantly greater in the offspring of trees selected for growth than in the offspring of unselected control trees (Fig. 4D, E). Differences between the offspring of selected and control trees were highly
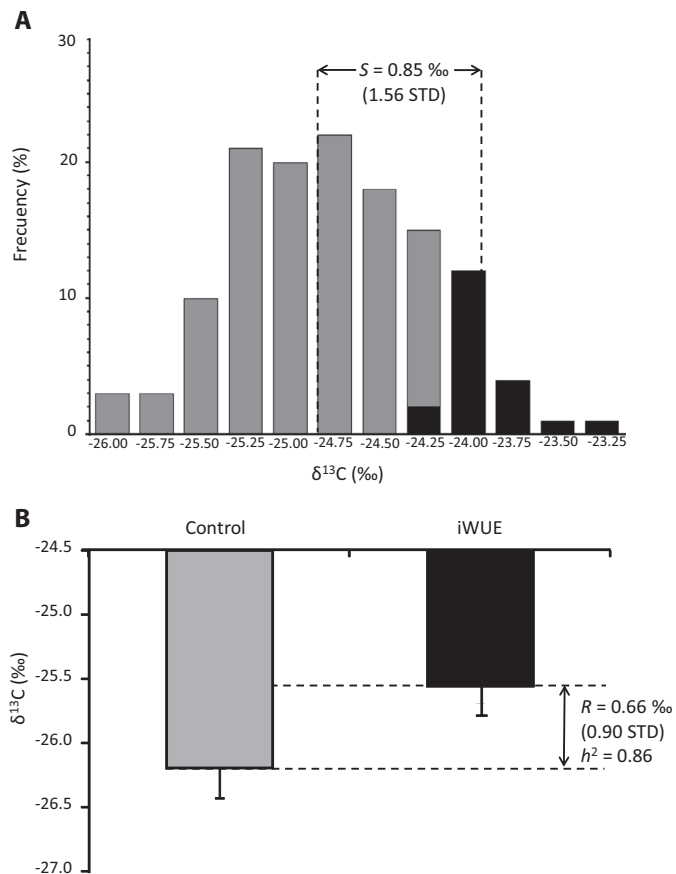
consistent across sites (no significant selection × site interaction, Table 1). Mono- and sesquiterpenes tended to show higher concentrations in Montañana than in Farasdués (Table 1). Neither selection nor test site significantly affected seedling survival (Table 1).

Comparison with the local seed source

The offspring of the unselected control trees had lower $\delta^{13}C$ than in the local seed sources, but no differences were observed between the offspring of trees selected for iWUE and the local seed source (Fig. 4C). Monoterpene concentration was significantly lower in the

TABLE 1. Summary of linear mixed-effects models showing the effects of selection (Growth vs. Control and iWUE vs. Control), test site and their interaction on survival, height, needle $\delta^{13}C$, and mono-, sesqui-, and diterpenes in the offspring population established in two progeny trials (Farasdués and Montañana). *F* ratios with their degrees of freedom and the associated probability ($P > F$) are shown. Significant effects ($P \leq 0.05$) are shown in bold.

| Variable	Selected for Growth vs. Control						Selected for iWUE vs. Control					
	Selection (SEL)		Site		SEL × Site		Selection (SEL)		Site		SEL × Site	
	$F_{1,13}$	$P > F$	$F_{1,13}$	$P > F$	$F_{1,13}$	$P > F$	$F_{1,13}$	$P > F$	$F_{1,13}$	$P > F$	$F_{1,13}$	$P > F$
Survival	0.8	0.382	1.0	0.331	0.7	0.403	0.9	0.363	1.6	0.232	0.1	0.823
Height	0.2	0.665	24.8	**< 0.001**	0.0	0.884	0.6	0.567	16.9	**0.002**	1.9	0.196
$\delta^{13}C$	0.8	0.388	5.3	**0.039**	0.7	0.414	9.4	**0.009**	2.8	0.116	0.1	0.746
Monoterpenes	6.8	**0.022**	4.4	0.057	0.1	0.763	0.2	0.695	5.6	**0.033**	0.0	0.945
Sesquiterpenes	5.1	**0.041**	2.6	0.129	0.4	0.537	0.7	0.413	6.9	**0.020**	0.6	0.443
Diterpenes	3.3	0.097	0.0	0.994	0.0	0.933	0.3	0.580	0.2	0.693	0.1	0.757
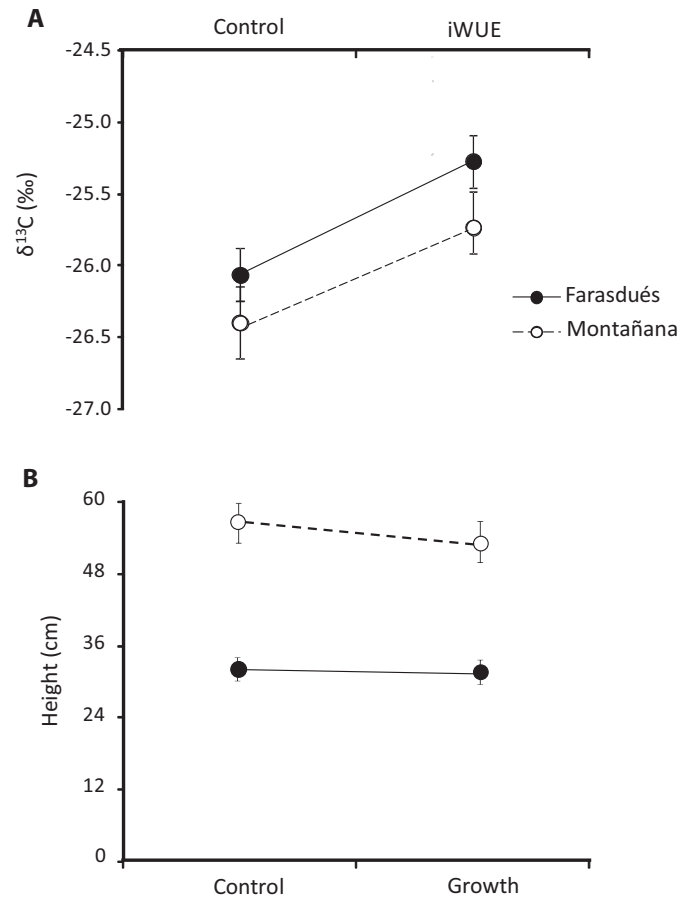
FIGURE 3. Direct response to selection for (A) intrinsic water-use efficiency iWUE and (B) growth in the offspring population established in the Farasdués (black dots and continuous line) and Montañana (open circles and dashed line) progeny trials. Mean ± SE (*N* = 8 composite samples for $\delta^{13}C$ and 48 individual seedlings for height) are shown.

local seed source than in the offspring of the experimental parental population, irrespective of the selection applied (Fig. 4D). For the remaining traits, the offspring of control and selected trees performed similar to the local seed source (Fig. 4A, B, E), except in the case of diterpenes, which showed a higher concentration in the offspring of trees selected for growth than in seedlings of the local seed source (Fig. 4F).

DISCUSSION

The results suggest that ($\delta^{13}C$-based) intrinsic water-use efficiency (iWUE) has high potential for evolutionary change in Aleppo pine in response to selective pressures imposed by increasing drought stress associated with climate change. The absence of correlated changes in other functional traits such as growth or defensive investment suggests that this evolutionary response for iWUE has few consequences on other traits. Similarly, selection for high growth rates, a key objective of most breeding programs, would have no negative impacts on other traits, at least at early stages of development.

Direct responses to selection for iWUE

The offspring of trees selected for enhanced iWUE also showed increased iWUE (i.e., high needle $\delta^{13}C$). This result reflects a strong direct response to selection and high realized heritability of iWUE-related traits. Response to selection for iWUE resulted in a high estimate of realized heritability ($h^2 = 0.86$). Our somewhat arbitrary choice of coefficient of relatedness (set to 1/3 for our open-pollinated seeds collected from different mother trees) affects this heritability estimate. Although it is common practice to account for the (typically unknown) contribution of full sibs within open-pollinated families (e.g., Hernández-Serrano et al., 2014), the coefficient chosen could be inaccurate for our study case. Due to the proximity between maternal trees and the small size of the parental population, a high proportion of full sibs, or even selfing, could have occurred within the seeds collected in each mother tree. Future studies using molecular tools for genotyping will permit pedigree reconstruction and, therefore, more accurate estimates of realized heritability of iWUE (Gaspar et al., 2009).

Other potential influences on the heritability estimates were (1) sampling for iWUE was performed in different tissues in the parental and offspring populations (wood and needles, respectively) (Klein et al., 2005; Hommel et al., 2014), (2) parental and offspring populations differed considerably in age at sampling, potentially leading to ontogenetic interferences (Castillo et al, 2018; Fonti et al., 2018; Cavender-Bares and Bazzaz, 2000), and (3) parental and offspring populations lived under different environmental conditions, possibly also contributing to different range scales of $\delta^{13}C$ (Cernusak et al., 2013). Although we attempted to minimize the bias from this sampling variation by standardizing the variable separately for the parental and the offspring population, the realized heritability reported here for
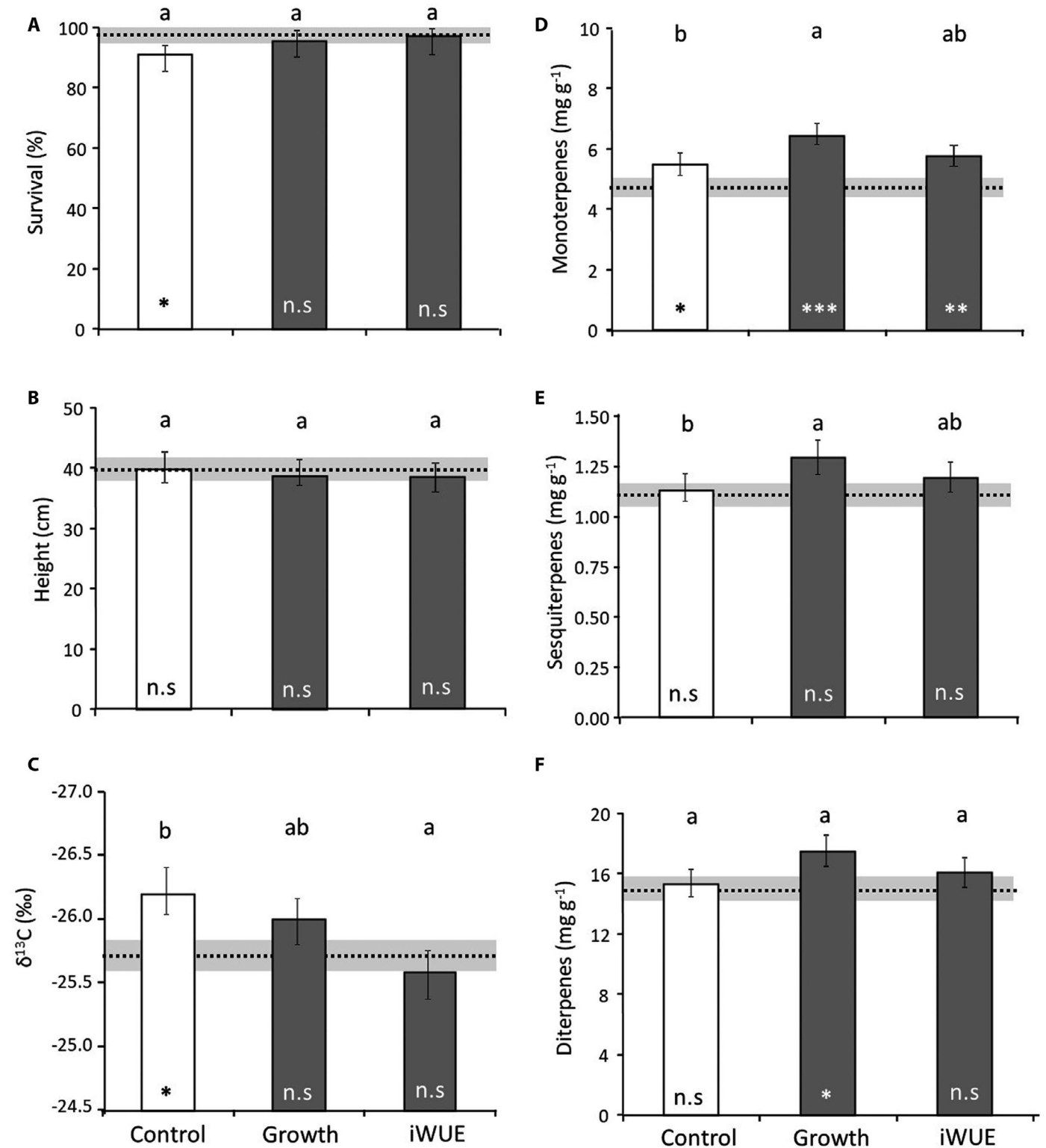
FIGURE 4. Mean ± SE of (A) survival, (B) height, (C) δ¹³C in needles, and (D) mono-, (E) sesqui-, and (F) diterpenes in needles of the offspring of control trees (white bars) and trees selected for growth and intrinsic water-use efficiency (iWUE) (gray bars). (*N* = 16 composite samples except for height, for which *N* = 96 individual plants). Performance of local seed source is shown by the dashed line (mean) and horizontal gray bands (±SE). Letters above bars indicate significant differences at $P \leq 0.05$ between the three seedlots (control, growth, and iWUE). Asterisks inside bars indicate significant differences (n.s. = nonsignificant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) between each seedlot and the local seed source.

iWUE should be taken with caution. Accuracy could be improved by future sampling schemes using wood as a more integrative tissue of iWUE at offspring ages similar to that of the parental population and by pedigree reconstruction of the offspring through genotyping (Gaspar et al., 2009).

Nevertheless, large additive genetic variances and moderate to high heritability estimates of $\delta^{13}C$ have been documented for pine species (Marguerit et al., 2014; Castillo et al., 2018). For example, heritability estimates for the taxonomically close Mediterranean pine *Pinus pinaster* ranged from low-moderate (0.17 ± 0.06 [Brendel et al., 2002] or 0.21 ± 0.10 [Lamy et al., 2011]) under mesic conditions to high (0.66 ± 0.25 [Corcuera et al., 2010] or 0.69 ± 0.31 [Aranda et al., 2009]) under xeric conditions. The range of reported heritability estimates is therefore considerable, perhaps because of methodological limitations associated with the trait itself, such as limited sample size due to high costs, the plant tissue analyzed (Klein et al., 2005), or the existence of ontogenic effects (Tanaka-Oda et al., 2010), in addition to differences in testing conditions. Nevertheless, the limited information available on the stability of genotypic ranking for $\delta^{13}C$ in forest tree species suggests consistency across years (e.g., for *Populus* spp.; Dillen et al., 2011). Moreover, for Aleppo pine under semi-arid and irrigated conditions, tree-ring and needle $\delta^{13}C$ proved to be equally good indicators of integrated leaf gas-exchange properties (Klein et al., 2005).

Despite the methodological issues described above, the direct response to selection observed in our study provides empirical evidence that selection for iWUE can be transmitted to future generations in natural populations of Aleppo pine. Whenever increasing drought stress associated with climate change imposes a selective pressure on Aleppo pine populations, an evolutionary change toward greater iWUE should be expected. This result is consistent with the high degree of climate-related among-population differentiation in iWUE in Aleppo pine (Voltas et al., 2008). Populations of this species originating from drought-prone environments show greater iWUE, preferential access to deeper water sources and a lower growth rate than populations from wetter origins, probably as a result of evolutionary responses to water scarcity (Voltas et al., 2015). This idea is supported by comparing the selected and control seedlots with the local (drier) seedlot, which showed greater iWUE than the offspring of the unselected parental population but similar values to the offspring of trees selected for iWUE.

Direct response to selection for growth

In contrast to $\delta^{13}C$, no significant direct response to selection for height growth was observed. This result was unexpected, as height growth in pine trees is known to be heritable (Kroon et al., 2011), and there is ample evidence of direct responses to selection for growth in forest trees (Liziniewicz and Berlin, 2019). In particular, results from breeding programs designed to improve tree growth and timber quality have documented large direct responses to selection for growth in many conifer species (Jansson, 2007; Burdon et al., 2008; Haapanen et al., 2016), including Aleppo pine (Matziris, 2000). A number of non-mutually exclusive reasons may explain our unexpected result.

Lack of response to selection for growth can arise from insufficient selection intensity or limited genetic variability in the parental population (Cornelius, 1994). Assuming the typical values of heritability for growth in pine trees ($h^2 = 0.1–0.3$) (Kroon et al., 2011), the selection intensity applied here (15%) may not be high enough to detect a significant response (Cornelius, 1994). In addition, not all of the 20 trees selected for their greater stem diameter were among the 20 tallest (see Fig. 1A), and therefore, the selection intensity was lower for this trait than for $\delta^{13}C$. The lack of response could also be explained by a poor phenotype–genotype relation in the parental population (i.e., preponderance of environmental effects). It cannot be ruled out that microenvironmental differences within the stand, typically large in forest ecosystems (Magnussen, 1993), have influenced tree growth and, therefore, phenotypically biased selection for highest growth (Zas, 2008). In addition, although the parental population was a coetaneous stand, there was some variation in tree age. Slight variations in individual tree age at relatively young stages (13 years old) could also have negatively impacted the effectiveness of the selection.

Heritable nongenetic factors such as maternal environmental effects and seed provisioning could also have contributed to the observed lack of direct response to selection for growth (Danchin et al., 2011). In pine trees, both of these factors are known to significantly affect early seedling performance beyond the genetic contribution of the mother tree (Zas et al., 2013; Suárez-Vidal et al., 2017). While few environmental differences should be expected among mother trees, large differences among trees in seed size, typically observed in pine trees (Wang and Ives, 2017), probably occurred. Differences among mother trees in seed provisioning can easily result in different early growth rates (Zas et al., 2013; Suárez-Vidal et al., 2017), and these effects may bias the comparison of the offspring of selected and unselected mother trees. In any case, this type of transgenerational plasticity tends to disappear with time since germination (Elwell et al., 2011). Future assessments of tree growth at older ages will help to determine whether selection for growth in the parental population will have any evolutionary effect on the offspring growth trajectory.

Correlated responses to selection

We expected to detect correlated responses to selection in nontarget traits because traits do not act independently and can be genetically linked (Walsh and Lynch, 2018). Santos del Blanco et al. (2015) found that selection for growth in *Pinus pinaster* Ait. negatively affected reproductive traits in the offspring of selected trees. In contrast, our results showed that selection for iWUE did not affect growth, and vice versa, at an early age. However, growth and defense allocation showed some linkage because of the positive effect on volatile terpene concentration detected in the offspring of trees selected for growth.

Theory predicts that life-history traits (i.e., growth, reproduction, and defenses) should trade off among each other because they share and compete for the same resources (Agrawal et al., 2010; Saeki et al., 2014). Previous reports of genetic correlations between growth and defensive investment in conifer trees range, however, from negative (e.g., de la Mata et al., 2017) to positive (Méndez-Espinoza et al., 2018). Environmental context dependency (Sampedro et al., 2011; Abdala-Roberts et al., 2014; Moreira et al., 2015) and genetically based differences (e.g., among-population differentiation in the genetic linkage between traits, Vázquez-González et al., 2020) may explain this lack of consensus. While harsh environmental conditions at the test sites should have elicited the emergence of trade-offs, the response to selection observed suggests that growth and chemical defenses are not genetically compromised in Aleppo pine, at least at intrapopulation level.

Similarly, although positive genetic correlations between iWUE and growth are common in woody plants (Fardusi et al., 2016), negative associations have also been reported for some forest species (e.g., *Pinus ponderosa* Douglas., Kerr et al., 2015) and, especially, for Mediterranean trees such as Aleppo pine (Voltas et al., 2008). Drought tolerance in isohydric pines like Aleppo pine is mainly based on tight stomatal regulation of water loss in response to drought (Klein et al., 2013). Stomata closure constrains gas exchange and may therefore directly impair primary production (Brodribb, 2009). Based on the results presented here, however, these physiological relationships do not appear to hold at the additive genetic level and would therefore have no evolutionary implications, at least at juvenile stages. Further sampling schemes using integrative wood samples and individual tree analysis are needed for more definitive conclusions.

Selection experiments with forest trees empirically testing direct response to selection for traits other than growth and wood quality are rare (MacLachlan et al., 2017; Liziniewicz and Berlin, 2019). In particular, we are not aware of selection experiments reporting correlated responses in functional traits related to tolerance to biotic and abiotic stresses in response to selection for water-use efficiency. Based on the results presented here, we conclude that natural selection for higher intrinsic water-use efficiency has the potential to produce considerable evolutionary changes in future generations, as evidenced by a single artificial selection event. Aleppo pine populations may have the evolutionary capacity to respond to increasing drought stress associated with global change in the short term. However, future studies should assess in detail the functional and fitness advantages of the changes observed and accurately determine the heritability of these transgenerational changes. Additionally, the lack of negative correlated responses to selection for water-use efficiency suggests that adaptive responses to drought stress would not compromise other relevant functional traits in Aleppo pine. We found no evidence that selection for growth, one of the main objectives of pine breeding programs, has negative effects on iWUE or chemical defense allocation at juvenile stages. Further research is needed, however, to determine whether the patterns observed are maintained at older ages and to study the correlated responses in other relevant life-history traits, such as those related to reproduction or storage.

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AUTHOR CONTRIBUTIONS

L.S., R.Z., J.C. and J.V. conceived and designed the experiment. J.C., E.S., L.S., R.Z., and E.S.V. carried out the assessments on the parental population. J.C. and J.V. performed the selections on the parental population. J.V. prepared the experimental design of the progeny trials. E.S.V., E.S., and E.N. conducted the field assessments on the young plantations. E.S.V. carried out the lab analysis. R.Z., J.V., and E.S.V. performed the statistical analyses. E.S.V. wrote the first draft of the manuscript, supervised by R.Z. All authors discussed the results and contributed to improvements and the final version.

DATA AVAILABILITY

Phenotype data and scripts are available at DIGITAL.CSIC repository (http://dx.doi.org/10.20350/digitalCSIC/12603).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Bole resin flow determination in the parental population.

APPENDIX S2. Nonvolatile resin estimation in the parental population.

APPENDIX S3. Total polyphenol estimation in the parental population.

APPENDIX S4. Summary of the phenotypic differences in growth, water-use efficiency, and chemical defences between the parental trees artificially selected for growth and water use efficiency, and the unselected control trees.

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