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Rapid changes in plasticity across generations within an expanding cedar forest

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Abstract

We investigated the inter-individual variation of phenotypic plasticity and its evolution across three generations within an expanding forest. Plasticity was assessed *in situ* from dendrochronological data as the response of radial growth to summer rainfall. A linear mixed model was used to account for spatial effects (environment, stand structure), temporal factors (stand dynamics) and the variation with age. Beyond these effects, our results reveal a significant inter-individual variance of growth and plasticity within each generation. We also show that the means and variances of growth and plasticity changed significantly across generations, with different patterns for both traits. The possible environmental and genetic drivers of these changes are discussed. Contrasting with the trade-off between stress tolerance and plasticity generally observed among populations, we detected a positive covariance at individual level, which does not support the cost of plasticity hypothesis in this case.

Keywords

phenotypic plasticity, micro-evolution, adaptation, dendrochronology, tree, Cedrus

Introduction

Rapid climate change represents an evolutionary challenge for species and communities. Long-lived organisms like trees will experience durable change of their environment within a single generation time and significant environmental shift in few generations. Phenotypic plasticity, adaptive capacity and migration potential will determine the response of the forests to these changes. Firstly, phenotypic plasticity (*i.e.* the environmentally induced phenotypic variation of a given genotype) determines the response of the current tree generations to the experienced changes. Secondly, once acclimatized populations survive and regenerate, further genetic evolution may proceed through selection: Pigliucci & Murren (2003) called "genetic assimilation" this process that turns a plastic response into genetic variation under permanent environmental shift. Evidence now increases that tree populations are capable of rapid adaptation, *i.e.* genetic evolution, in few generations (Daubree & Kremer, 1993; Skrøppa & Khomann, 1997; Rehfeldt *et al.*, 2001). Such genetic shifts rely on the genetic variation that is maintained within populations and, among other processes, genetic variation can be maintained thanks to GxE interactions combined with gene flow across environments, either spatially or temporally (Gillespie & Turelli, 1989; Ellner & Hairston, 1994). Thirdly, migration takes several generations to adjust the geographical range of a species following the movement of its fundamental or potential niche (Higgins *et al.*, 2003; MacLachlan *et al.*, 2005). Adaptation and migration have specific interactions in the context of rapid environmental change (Davis & Shaw, 2001; Savolainen *et al.*, 2007): rapid environmental change increases the adaptational lag (the departure from optimum of the actual response to local environment) which may reduce the migration load (the chance of an immigrant being less fit in the receiving population).

Much work has been devoted to phenotypic plasticity and its ecological consequences (Miner *et al.*, 2005) but understanding patterns of selection for individual phenotypic plasticity within

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populations under environmental change still deserves attention (Pigliucci, 2005). Few studies have been devoted to the individual level of phenotypic plasticity within wild populations (Brommer *et al.*, 2005; Nussey *et al.*, 2005; Pigliucci, 2005; Nussey *et al.*, 2007; Charmantier *et al.*, 2008) and, to our knowledge, none in forest trees.

Among tree populations, elevational or latitudinal clines are frequently observed, which are interpreted as the result of selection and local adaptation processes (Eriksson *et al.*, 1980; Mikola, 1982; Ducousso *et al.*, 1996). The adaptive traits that show strong clinal variation among populations also show an important genetic variation within populations (Kaufman & Smouse, 2001; Savolainen *et al.*, 2004). Based on common garden experiments, clinal patterns of variation for phenotypic plasticity at population level were also evidenced in trees: latitudinal and longitudinal clines of reaction norms (Matyas & Yeatman, 1992; Rehfeldt *et al.*, 2001; Rehfeldt *et al.*, 2002) and elevational clines of GxE interaction (Modrzynski & Eriksson, 2002). By contrast, in a model based approach that compared the response to climatic variations *in situ*, Chuine *et al.* (2000) hardly detected significant variation among populations for only one species out of five. Schlichting (1986) pointed out the fact that, theoretically, the variation for a quantitative trait and GxE interaction can evolve independently or, in other words, that selection can equally change the height and the slope of a linear reaction norm. However, dependence between traits and plasticity was experimentally observed by Rehfeldt *et al.* (2001): tested in cold conditions, *Pinus contorta* populations from higher latitudes grow better than populations from lower latitudes, but they grow worse in a favourable environment where temperature is not limiting and, therefore, are less plastic. Similarly, Modrzynski & Eriksson (2002) observed that *Picea abies* populations from higher elevation have a better resistance to early frost, in terms of bud-set phenology, and have a lower plasticity because their phenological response does not change when drought stress is applied as it does for lower elevation populations. The trade-off between local adaptation to harsh conditions and plasticity at population level may result from an intrinsic cost of phenotypic plasticity (Relyea, 2002), or from a selection for higher responsiveness in favourable environments. The first hypothesis would also result in a negative correlation between the response to stressful conditions and plasticity at individual level within population, which is not necessarily the case for the second hypothesis.

To assess the diversity of phenotypic plasticity at individual level and investigate its short term evolution within tree populations, we conducted a retrospective micro-evolution study within an introduced population that experienced continuous environmental changes and presents an interesting demographic structure in semi-discrete generations. We studied the variation of phenotypic plasticity under the reaction norm approach (Via et al., 1995), assuming a linear model of the phenotypic response in terms of annual radial growth to a continuous environmental parameter, summer rainfall. Using increment cores we retrospectively obtained numerous (up to 90) observations for each individual in contrasted environmental conditions, leading to individual measures of plasticity. We also estimated the covariance between the height and slope of linear reaction norms at individual level.

Transplanted forests from the 19th and 20th century represent a unique experimental opportunity to perform micro-evolution studies in trees through retrospective approaches (Zheng & Ennos, 1999). Indeed, populations that were transplanted out of their natural geographical range can inform us on the impact of evolutionary forces that shape the diversity after rapid environmental change: demo-genetic processes and adaptation. Cedar (*Cedrus atlantica* Manetti) had been introduced in France from Algeria mid 19th. The Luberon cedar forest is located in the South East of France, under Mediterranean climate, at 700m elevation. Originally planted in 1863 to stop erosion in over-grazed areas (Cointat, 1996), cedar has naturally regenerated without any silvicultural management until the 1990's. The species has been transplanted from Southern to Northern latitude (from Algeria to France). However, initial conditions in the transplantation site were particularly severe for *Cedrus*: bare and thin soil, summer drought climate and strong dry northern wind (mistral). Thus, initial mortality was high and the density of surviving founder trees was as low as 8 trees/ha as revealed in an aerial picture dated 1939 obtained from the Institut Géographique National. These founder trees produced many seeds and a closed forest was formed at the next generation, which now provides much more favourable conditions for seed germination and seedling survival during summer (density exceeding 50 seedlings.m^{-2} has been observed in a regeneration plot 8-years after germination, F. Courbet pers. comm.). Therefore, we hypothesize that a strong initial selection for drought resistance has shaped the founder gene pool and, for the next generations, that strong selection for competing ability occurred within dense seedling patches. Three generations, or more precisely cohorts, of trees are clearly identified today, including the founder trees. In a previous paper, we showed that population admixture had occurred in the planting stock and that hybridisation among initial gene pools started as soon as the first regeneration phase on site (Lefèvre *et al.*, 2004). We observed no severe genetic erosion, neither for neutral nor non-neutral genes, and we concluded that this population has experienced intensive re-organisation of the diversity and not only genetic erosion (Fallour *et al.*, 2001; Lefèvre *et al.*, 2004).

Trees are long-lived sessile organisms and their wood provides a record of the plastic response to the annual change of their environment. In this study, we assessed phenotypic plasticity as the response in terms of radial growth to annual climatic stochasticity through a dendrochronological approach, which retrospectively provides growth data of individual trees at different ages in their life. Temporal changes of environment include annual and long term climatic variation, as well as the evolution of stand structure. The impact of these temporal factors is partly confounded with an internal age effect. We used a global model of analysis that accounted for these different factors and their interactions: climate, tree age and year, as a surrogate for other climatic variation and evolution of stand structure. Our goals in this study were to (i) estimate the variance of phenotypic plasticity at individual level within the forest, (ii) investigate the changes in growth and plasticity among successive generations in various environmental conditions within the forest and (iii) investigate the relationship between growth performance and plasticity at individual level.

Materials and methods

Tree sampling

The cedar forest on Mount Luberon developed from 3 initial plantations with a distance of about 1500m between each of these areas. The population has expanded through natural regeneration and currently forms a closed forest of ca 300ha. In 1995, we had sampled 197 trees in 3 zones of the forest, *ca*. 8-10ha each, that correspond to the initial plantation areas. An aerial picture dated 1939 showed that initial mortality has been much lower and density much higher in zone #3 compared to zones #1 and #2. Soils conditions are homogeneous on a large scale throughout the forest (calcareous lapiaz), but soil depth varies locally at very small scale (Fallour, 1998).

Sampled trees belonged to 3 different age classes as described in a previous publication (Lefèvre *et al.*, 2004): G0, the surviving founder trees issued from seeds collected in the natural range, G1, the first generation that appeared on site from intercrossing among G0 trees and G2, the second generation that appeared when G1 trees became fertile (G0 still there). Sampled trees were individually mapped within each zone: successive generations slightly differ in their spatial distribution with a clearly clustered pattern within generation and repulsion between generations (Fig.1).

Radial growth and age determination

For each tree, we extracted three increment cores by means of a Swedish increment borer, one at the northern side of the tree and the others oriented at 120∞ each. Because cedar trunks often had an asymmetric shape due to the strong northern wind, the north side core had the highest probability to reach the pith. Increment cores were taken at 60 cm above ground level, which was the minimum height possible when using this borer. Increment cores were immediately smoothed with a plane after extraction and stored in dry condition under a press until analysis. Cores were visually crossdated under a stereomicroscope in order to find missing rings or false rings (Stokes & Smiley, 1968; Schweingruber *et al.*, 1990). Then, annual ring widths were measured using an Eklund measuring device within 10^{-2} mm precision. After measuring radial series, crossdating quality was checked by comparing the visual agreement between plotted ring-width series and by calculating correlation coefficients between ring-width series. Thus, our temporal calibration of ring-width series was not sensitive to the estimation of age, which allowed using all trees simultaneously to estimate the mean effect of each year on radial growth (*Year* effect in the statistical analysis below).

For most (>80%) of the trees, at least one of the three cores included the pith. For the others, the number of inner rings missing in the core was estimated from the curvature of the innermost rings according to the geometric method developed by Duncan (1989). To assess the age of each tree, we estimated the number of invisible annual rings at 60 cm height on the basis of the observation of young seedlings grown in different environments in the forest which age could be precisely estimated from their branching architecture (data not shown). Thus, we added 10 years for the G1 and G2 trees, and 15 years for the founder G0 generation that grew in harsh conditions, which finally gave an estimated date of species introduction that agrees with written historical records.

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Climatic data

The climate series were obtained from Météo France. They had to be sufficiently long and had to be situated as close as possible to the study area for the analysis of climate-growth relationships. The best series available were obtained from the meteorological station at Avignon, located at 50m above sea level, which provided mean monthly maximal and minimal temperature as well as monthly precipitations for the whole study period that we restricted to the period 1904-1994 in order to avoid missing data. Avignon and the Luberon mountain differ in altitude but they belong to the same climate sub-unit according to monthly precipitation patterns as defined by Guiot (1986).

Under Mediterranean climate, rainfall is expected to be the main limiting factor for tree growth on thin soils. A previous analysis on the period 1921-1979 (Guibal, 1985) revealed that, among several climatic parameters, rainfall in spring and summer were generally the best predictors of annual ring-width for cedars, thus showing that water supply was the limiting factor for radial growth. Therefore, we computed cumulative rainfall from May to August (*MJJA*) as a yearly climatic parameter. It varied from 16mm to 421mm during the study period. Note that some statistics described below refer to the theoretical value $MJJA=0$ which represents stressful conditions that still remain ecologically sound, where no rainfall would occur during this 4-months period regardless of the rainfall during the rest of the year.

May to August rainfall was comparable among generations (Fig. 2). Indeed, there was no significant trend for the *MJJA* climatic variable over the study period 1904-1994 (Pearson's correlation between *MJJA* and year was $r=0.016$, $P_{value}= 0.88$). Furthermore, there was no difference in average *MJJA* among the three periods 1904-1920 (G0 trees alone), 1921-1950 (G0 and G1 trees) and 1951-1994 (all three generations; ANOVA test $F_{2,88}=0.83$, $P_{value}=0.44$).

The first period showed a slightly higher variance among years but this was not significant (Levene's homogeneity of variance test, $F_{2,88}=2.82$, $P_{value}=0.064$).

Statistical analysis

For each tree, we computed annual radial growth increment (RGI) as the mean of annual ring-width by averaging the 3 core chronologies. Phenotypic plasticity was defined as the linear response (slope) in RGI to summer rainfall. A linear mixed effect model was used where RGI varied among zones, generations and years, depending on the age of trees and summer rainfall (MJJA) as covariates. A random effect of individuals within each zone and generation was also included. The variation of plasticity among zones and generations, but also among individuals within each generation, was directly investigated within the model by the use of interaction effects implying MJJA. Thus, the terms included in the global model were as follows:

- *Zone* (α , fixed): large scale spatial heterogeneity of environmental condition (topography and stand dynamics) in different parts of the forest;
- *Generation* (β , fixed): a genetic effect partly confounded with a micro-environmental effect because of different spatial distributions of generations within each zone;
- *Zone x Generation* ($\alpha\beta$, fixed): the interaction among previous effects;
- *Age* (covariate): we assumed a quadratic effect of age to account for the juvenile and senile phases of RGI, which was not corrected for tree diameter prior to the analysis;
- *MJJA* (covariate): a linear effect of summer rainfall on radial growth (it is sufficient to consider a linear rather than a quadratic relationship because water availability is a limiting factor for growth in the Mediterranean);
- *Year* (δ , fixed): a categorical variable beside the climatic covariate *MJJA* to account for other annual climatic variability and all other temporal variations, in particular the evolution in stand structure (tree density);

- *MJJA x Zone* and *MJJA x Generation* (fixed): interaction terms accounting for the difference in plasticity among zones and among generations;
- *MJJA x Generation x Zone* (fixed): variation among zones of the generational differences in plasticity;
- *MJJA x Age* and *MJJA x Age*^{2} (fixed): variation of the response to climate with age, constant among zones and generations;
- *Individual* (random): variance of growth among individual trees within each zone and generation; differences among generations of inter-individual variances were allowed;
- *MJJA x Individual* (random): variance of phenotypic plasticity among individual trees within each zone and generation; differences among generations of inter-individual variances were allowed;
- a covariance between the *Individual* effect and the *MJJA x Individual* effect was estimated within each generation.

We also investigated the presence of a spatial structure in the *Individual* effects, and whether the variance among individuals and the spatial structure varied among generations (generation as a GROUP of the RANDOM effect in SAS).

Finally, we included a temporal covariance structure in the residuals to account for the intra-individual correlations among observations. We used a first-order autoregressive model, AR(1), where the correlation between two residuals of a given individuals decreases with the lag *t* between these observation as a function corr(t)=ρ^t. The correlation between two residuals of two different individuals equals zero. The auto-correlation parameter ρ was estimated jointly with all other parameters. A positive value of ρ may be due to an "after-effect" of any single event over several consecutive years of growth. A negative auto-correlation ρ is expected when high and low growth alternate in successive years.

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The overall model can be written as follows:

$$RGI_{z,g,i,y} = m + \alpha_z + \beta_g + (\alpha\beta)_{z,g} + h_1 Age_{z,g,i,y} + h_2 Age^2_{z,g,i,y} + fMJJA_y + \delta_y + j_z MJJA_y + g_g MJJA_y + (jg)_{z,g} MJJA_y + (fh_1)_{yi} MJJA_y.Age_i + (fh_2)_{yi} MJJA_y.Age_i^2 + A_{i(z,g)} + B_{i(z,g)} MJJA_y + E_{z,g,i,y}$$

where *z, g, i, y* respectively stand for *Zone*, *Generation*, *Individual* and *Year*, and where:

$\mathrm{var}(A_{i(z,g)}) = \sigma^2_A(\mathrm{g})$ is the inter-individual variance of annual growth in stressful conditions (RGI, predicted at $MJJA=0$), potentially varying among generations;

$\mathrm{var}(B_{i(z,g)}) = \sigma^2_B(\mathrm{g})$ is the inter-individual variance of phenotypic plasticity (PP), potentially varying among generations;

$\mathrm{cov}(A_{i(z,g)}, B_{i(z,g)}) = \sigma_A(\mathrm{g})\sigma_B(\mathrm{g})\rho_{AB}(\mathrm{g})$ is the inter-individual covariance between growth and plasticity, this parameter was assumed to vary among generations; this covariance was also used to compute the inter-individual variance of annual growth in favourable conditions (RGI, predicted at $MJJA=350$) as follows: $\mathrm{var}(A_{i(z,g)}) + 350^2\mathrm{var}(B_{i(z,g)}) + 700\mathrm{cov}(A_{i(z,g)},B_{i(z,g)})$;

$\mathrm{var}(E_{z,g,i,y}) = \sigma^2_E$ is the residual variance within individual and year, the temporal structure of the residuals being:

$$\mathrm{corr}(E_{z,g,i,y}, E_{z',g',i',y'}) = \rho^{|y-y'|} \quad \text{if } z=z', g=g' \text{ and } i=i'$$

$$= 0 \quad \text{elsewhere}$$

Analyses were conducted with PROC MIXED (SAS Institute) using the REML method. We systematically used type III tests to eliminate partially confounding fixed effects, except for *MJJA* for which we used type I test adjusted for *Zone*, *Generation, Zone x Generation, Age* and *Age²* because this would be totally included within the *Year* effect in a type III test. In order to achieve a robust test of fixed effects, we used the Satterthwaite correction to compute the degrees of freedom (Littell *et al.*, 2006). Note that tests of fixed effects (such as *Generation*) refer to the difference among treatments for null values of the covariates and, therefore, to stressful conditions where *MJJA*=0.

Since trees from different generations both differed in age at a given year and experienced different stand structure or different climatic conditions at a given age, mean values per generation were not directly comparable due to confounding effects. Therefore, for each generation within each zone, we computed means of growth and plasticity adjusted for *Age*, at ages ranging from 10 to 100 years, and for *Year*, restricting to 1960-1994 that represents a period of stable stand structure shared by all trees from the three generations. We first obtained predicted growth values for each year and each age during this period using the estimated model. Then, we computed adjusted means of growth under stressful climate and plasticity respectively by the intercept and the slope of the regressions of predicted growth on corresponding values of summer rainfall; we then used these two parameters to compute adjusted means of growth in favourable conditions (*i.e.* value of *MJJA*=350). These adjusted means were comparable among generations.

Results

Variation patterns for radial growth increment (RGI)

Although many observations contributed to the analysis (12901), the global model accounted for a fair amount R^2=54% of total variation.

Differences in RGI among zones was highly significant (P_{value}<0.0001, Table 1). In particular zone #3 had a lower growth than zones #1 and #2. Globally, over the whole study period, we detected no difference among generations, neither for the whole forest nor within each zone (*Generation* and *Zone x Generation* effects non significant, Table 1). However, the comparison of growth potential among generations deserves more detailed analysis because, over the whole study period, they experienced different environmental changes at different ages.

Globally, there was a highly significant response of RGI to summer rainfall *MJJA* (P_{value}<0.0001, Table 1) that confirms the relevance of this climatic variable as a determinant for growth. Besides, the *Year* effect was highly significant (P_{value}<0.0001, Table 1), which reveals another prevailing environmental factor of temporal variation different from summer rainfall. This temporal *Year* effect is largely explained by the evolution of stand structure as can be seen from Fig. 2: see the much larger growth of G0 trees alone in the first years compared to juvenile growth of G1 and G2 individuals, see also the decrease of growth of G0 and G1 individuals while G2 trees emerged during the 1960-1975 period.

The quadratic effect of age on RGI was highly significant and provided a parabolic shape of radial growth with a maximum at ages 67, 61 and 58 for summer rainfall *MJJA* of 0, 200 and 350mm respectively.

We estimated the *Year* and quadratic *Age* effects from the global model and used these parameters to predict the RGI values that each generation would experience within each zone during any of the years within the period 1960-1994 of stable stand structure and where all three generations were effectively present. The same pattern was observed in all three zones: the G0 generation had the lowest predicted growth in stressful climate (*i.e. MJJA*=0) whereas the G2 generation had the highest predicted growth in favourable conditions (*i.e.* *MJJA*=350), the G1 generation was rather similar to G2 in stressful climate and to G0 in favourable conditions (Fig. 3). In zone #3, the difference between generations was larger for growth in stressful conditions but smaller for growth in favourable conditions (Fig. 3)

Inter-individual variance estimates for RGI at *MJJA=0* were not significantly different from 0 within any generation (Table 1). Inter-individual variance estimates for RGI at *MJJA=350* were almost 20-times higher, decreasing from G0 to G1 and increasing from G1 to G2.

No spatial structure of individual effects could be detected. This means that close individuals did not show correlated RGI and implies that the clustering of individuals of the same generation (see Fig.1) should not be responsible for the detected differences in mean RGI among generations.

Temporal autoregressive structure in the residuals was highly significant and the autocorrelation parameter estimates were positive within each generation (AR(1)=0.799 for generation G0, AR(1)=0.867 for G1 and AR(1)=0.756 for G2 with P_{value}<0.0001 in all cases). This temporal pattern was beyond the temporal trends included at higher level within the *Year* and *Age* effects.

Variation patterns for phenotypic plasticity

The response to summer rainfall varied with age in a quadratic way (*MJJA* x *Age* and *MJJA* x Age^2 effects both significant, P_{value}<0.0001, Table 1). Plasticity was maximum at intermediate age, *i.e.* in a multiplicative effect where growth variation due to climate was maximum when growth was maximum. There was a significant variation of plasticity among zones (different slopes in the different zones depicted by the *MJJA x Zone* effect,

$P_{value}<0.0001$, Table 1) and zone #3 was characterised by lower predicted values of plasticity for the 1960-1994 period (Fig. 3). Furthermore, the variation of plasticity among generations was highly significant (*MJJA x Generation* effect, tested for a value of *Age* = 0, $P_{value}<0.0001$, Table 1), even greater than variation among zones. This variation among generations was stable over the 3 zones of the forest (*MJJA x Zone x Generation* not significant, $P_{value}=0.091$, Table 1). The difference between generations for predicted values of plasticity during the 1960-1994 period resulted from the differences for RGI both in stressful climate and favourable conditions as previously mentioned. Predicted plasticity values increased from G0 to G2 in zone #1 and zone #2, mainly because of increased growth potential in favourable conditions. The situation was different in zone #3 where growth potential in stressful climate increased even more between G0 and G2, which finally resulted in similar values of plasticity between the two generations. The G1 generation had the lowest predicted plasticity due to a higher RGI in stressful climate than G0 and a lower RGI in favourable conditions than G2 (Fig. 3).

Inter-individual variance of plasticity was highly significant within G0 and G1, and it was marginally significant within G2 (*Individual* x *MJJA* variance, Table 1) . The inter-individual variance of plasticity slightly decreased from G0 to G1 and remained constant from G1 to G2. The goodness of fit of the regression of growth on summer rainfall for each individual tree varied from R^2= 0.1% to 33% in the raw data and from R^2= 2% to 63% when using growth values predicted by the global model (Fig. 4). As expected from the *Individual* x *MJJA* variance parameters, the fit was better for the younger generation G2.

Within each generation, the inter-individual covariance between growth (estimated in the model at *MJJA*=0) and plasticity was significantly positive and strongly increased from G0 to G2 (Table 1).

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Discussion

Over a short period of time (<5 to 10 years), the main environmental factor that determines the annual variation of radial growth is the climate. Over a longer period of time, evolution in stand structure and other ecological parameters, as well as the age of the trees must also be taken into account. In this work, we used dendrochronological data as long term temporal records of the response to annual climate variations. A large dataset was produced and a complex model of analysis was required to disentangle the various factors that determine the variation of radial growth increment (RGI), especially to adjust the data for the effects of age and large temporal scale stand structure before comparing generations.

Globally, beyond the effect of age, we found a significant response of growth to summer rainfall and longer term changes (respectively *MJJA* and *Year* effects). We detected a highly significant response to summer rainfall although this climate variable was measured in Avignon some kilometers away from the forest, at lower altitude: this reveals the good correlation of seasonal variations across years at this geographical scale. In the statistical model that we used, the discrepancy between summer rainfall in Avignon and the Luberon forest on particular years, *e.g.* due to local storms, was included in the *Year* effect. Therefore, although already highly significant, the actual plasticity of trees may be underestimated in our analysis and the differences among groups of individuals even larger than detected here. We found an AR(1) structure in the residuals that could be explained by a lag in the effect of annual climate on growth or by a failure of the model to explain perfectly the effect of age and year on RGI.

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Within the forest, the three sampled zones significantly differed for growth and plasticity. The lowest growth was found in zone #3 although the highest survival rate of the initial plantation had occurred in this area. This variation in growth performance among zones relates both to ecological heterogeneity as well as differences in stand structure and stand dynamics. After initial plantation in harsh conditions to which G0 trees had to survive, the environmental conditions have progressively changed since the establishment of the G1 generation: an organic layer has developed, a semi-closed stand structure has developed providing a shelter for young seedlings during hot and dry summer, the competition from herbaceous species has decreased, while the seed set and the competition among seedlings have increased. Higher initial survival rate in zone #3 resulted in higher density and higher competition in the area at generation G0, thus reducing both radial growth and its response to climate (plasticity) through a multiplicative effect.

Over the whole study period, there was no significant variation of radial growth under stressful conditions, neither among generations nor among individuals within generation. However, this is not direct evidence of the absence of genetic effects for growth because, during the entire period of time, the different generations did not experience the same stand structures at the same age. Indeed, the adjusted means of growth for the period 1960-1994 systematically increased from the G0 to G2 generation in each of the three zones, *i.e.* in three different environments, both under stressful and favourable climatic conditions (*MJJA*=0 and 350 respectively). Furthermore, in medium stress conditions (analysis with centred *MJJA*, data not shown), the *Generation* effect on RGI significantly increased from G0 to G2 even for the whole study period. Moreover, individual variance estimates in favourable conditions (*MJJA*=350) were significant within each zone and each generation for the whole study period and, finally, there was no spatial structure of the individual effects as would be expected if micro-environmental heterogeneity were the main driver of the inter-individual variance. These results strongly suggest that genetic variation exists for growth potential and that it increased in two generations, although it was masked in the global analysis by major environmental changes that occurred over the study period. The inter-individual variance for growth decreased from G0 to G1 and then increased from G1 to G2: such evolution was expected given the initial population admixture at G0 (Lefèvre *et al.*, 2004), forming a "hybrid generation" at G1, further segregating at G2.

Individual variance estimates for phenotypic plasticity were significant within each generation over the whole study period. The absence of spatial structure among individual effects is a clear indication that such variation among individuals within generations was not only due to micro-environmental effects but also, at least partly, under genetic control. The existence of genetic diversity for the plasticity of a trait not only has an impact on its response to selection when environment changes (Pigliucci & Murren, 2003; Crispo, 2007), but it also means that plasticity itself can evolve. Previously, by using common garden experiments of controlled crosses in eucalypts, Bouvet *et al.* (2005) had demonstrated that plasticity, defined as growth response to stand density, was under genetic control and could therefore respond to selection.

The unbalanced design was an unavoidable challenge for such *in situ* study of plasticity across several generations of trees: individuals from different generations did not experience the same stand structure at the same age. Therefore, we assumed that each zone and each generation respond similarly to age but can vary in growth and plasticity and we used type III tests to avoid confounding between *Age*, *Year* and *Generation* effects on growth and plasticity. We checked, by simulating data on the same unbalanced experimental design as here (data available as supplementary material), that the type III tests would not artificially lead to a significant difference of plasticity among generations when such difference does not exist. For example, decreased growth and/or increased plasticity with years (that could be

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related to the evolution of stand structure) did not lead to confounding between *Generation* and *Year* effects. The only simulation where non-existing difference of plasticity would appear more frequently than 0.05 was in the case of important spatial structure of growth and plasticity (confounded with spatial clustering of the generations), which was not the case here. Thus, based on the assumption of constant response to age, the adjusted means for the common period of stable stand structure 1960-1994 computed from the effects estimated over the whole dataset could be compared between generations.

We revealed significant changes of phenotypic plasticity across generations both over the whole period and the restricted period of stable stand structure. These changes did not simply result from a multiplicative effect as did the difference among zones: for example, the G1 generation had a higher growth in dry conditions than G0 but a lower plasticity and similar growth in favourable years. Three non-exclusive hypotheses could account for the variation of plasticity across generations: (i) an environmental effect on the response to age inducing a difference of this response among generations (that we assumed to be constant in our analysis), (ii) a genetic response to various selection pressure (selection for tolerance to drought stress in G0 *vs* selection for competing ability and higher responsiveness in G2) or (iii) a genetic change due to complex genetic architecture and segregation patterns following initial population admixture. The first hypothesis would represent a long-term record of environmental changes within the life-span of trees, the other two hypotheses would represent genetic evolution. The first two hypotheses depend on environmental conditions, and we would therefore expect different generational changes among zones, wheras the third hypothesis does not depend on environmental conditions and should have the same impact anywhere. Indeed, in all zones we observed a decrease of plasticity from G0 to G1, followed by an increase from G1 to G2, but the amount of increase from G1 to G2 varied among zones. Therefore we suspect that phenotypic plasticity has genetically evolved since the original admixture, with or without further response to selection. Contrasting with growth, the inter-individual variance of plasticity did not increase from G1 to G2 as expected from the admixture scenario alone, suggesting a stabilising selection constraint in G2. This would indicate different selection patterns for growth and plasticity in this forest. The environmental or genetic origins of the generational changes of plasticity still need further investigation, using *in situ* or common garden approaches.

The evolution of phenotypic plasticity raises the question of costs and limits of plasticity that has been treated theoretically more often than empirically (Relyea, 2002; Steinger *et al.*, 2003; Pigliucci, 2005). In this study, at individual level within each generation, we detected a positive covariance between plasticity and tolerance to drought stress. Interestingly, despite the positive covariance at individual level, we observed one case of antagonistic variation across generations: in zone #3, the G1 generation had a better growth in stressful conditions but lower plasticity than G0. More generally, the G1 and G2 generations did not differ for tolerance to drought stress but differed for plasticity. Therefore, the *Cedrus* case does not support the hypothesis of intrinsic cost of plasticity, it rather shows an increased responsiveness at the G2 generation. If selection has contributed to the change of plasticity across generations, the correlated increase in growth and plasticity from G1 to G2 would suggest a Baldwin effect of adaptive evolution through adaptive phenotypic plasticity rather than a genetic assimilation process with increased canalization (Crispo, 2007). In the absence of cost of plasticity at individual level, the balance between selection pressure for stress tolerance or competing ability in different environments could explain the trade-off between plasticity and stress tolerance generally observed among forest tree populations.

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Figure 1. Spatial distribution of sampled trees within the three studied zones in the forest

(G0 trees appear as "+", G1 trees as open circles, G2 trees as filled dots).

Figure 2. Annual variation of summer rainfall from May to August (*MJJA*) during the study period and mean radial growth increment (RGI) for each generation of trees

(dot line for generation G0, solid line for G1, bold line for G2)

Figure 3. Adjusted means of radial growth increment (RGI) under limited summer rainfall (*MJJA*=0mm) or high summer rainfall (*MJJA*=350mm) and phenotypic plasticity (PP) as quadratic functions of age (ranging from 10 to 100 years), for each generation in each zone during the period 1960-1994 of shared and stable stand structure. The model assumes the same quadratic effect of age for all generations and zones.

(dot line for generation G0, solid line for G1, bold line for G2)

Figure 4. Goodness of fit of the individual regressions of growth on summer rainfall within each zone and each generation

(a) distribution of the R^2 values of individual regressions on raw data

(b) distribution of the R^2 values of individual regressions on predicted values from the global model

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Table 1. ANOVA tests of fixed and random effects on radial growth increment.

Fixed effects	Num DF	Den DF	F	P_{value}
Zone	2	146	19.06	<0.0001
Generation	2	260	2.62	0.075
Zone x Generation	4	107	2.39	0.056
Age	1	533	29.89	<0.0001
Age^2	1	1273	76.58	<0.0001
MJJA	1	274	1618.02	<0.0001
Year	89	3818	131.62	<0.0001
MJJA x Zone	2	122	13.09	<0.0001
MJJA x Generation	2	149	50.59	<0.0001
MJJA x Generation x Zone	4	87.4	2.07	0.091
MJJA x Age	1	270	56.65	<0.0001
MJJA x Age^2	1	9261	256.60	<0.0001

Random effects	Level	Variance/Cov	P_{value}
Individual (at MJJA=0)	within G0	162.10	0.259
Individual (at MJJA=0)	within G1	0	-
Individual (at MJJA=0)	within G2	173.86	0.404
Individual at *MJJA*=350	within G0	3073	<0.0001
Individual at *MJJA*=350	within G1	2587	0.0003
Individual at *MJJA*=350	within G2	5847	0.0006
MJJA x Individual =PP	within G0	0.016	<0.0001
MJJA x Individual =PP	within G1	0.009	0.0003
MJJA x Individual =PP	within G2	0.010	0.045
Cov(Individual, PP)	within G0	1.329	0.051
Cov(Individual, PP)	within G1	2.180	0.016
Cov(Individual, PP)	within G2	6.329	<0.0001

Fixed effects were tested following type III tests, except summer rainfall covariate (MJJA) for which we used type I test to avoid complete confounding with Year effect. Variance components were estimated using REML. See text for further details on the model of analysis.

Figure 1

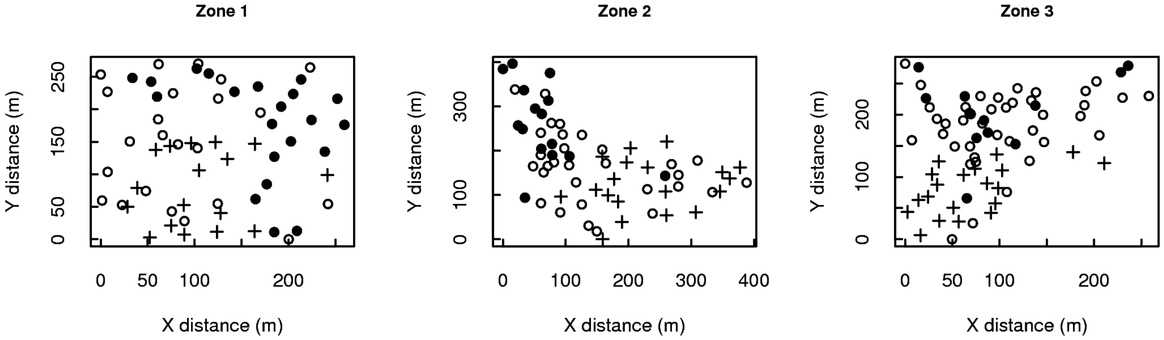

Zone 1
Zone 2
Zone 3
Y distance (m)
0
50
150
250
0
100
300
0
100
200
0
50
100
200
0
100
200
300
400
0
50
100
200
X distance (m)
X distance (m)
X distance (m)

Figure 2

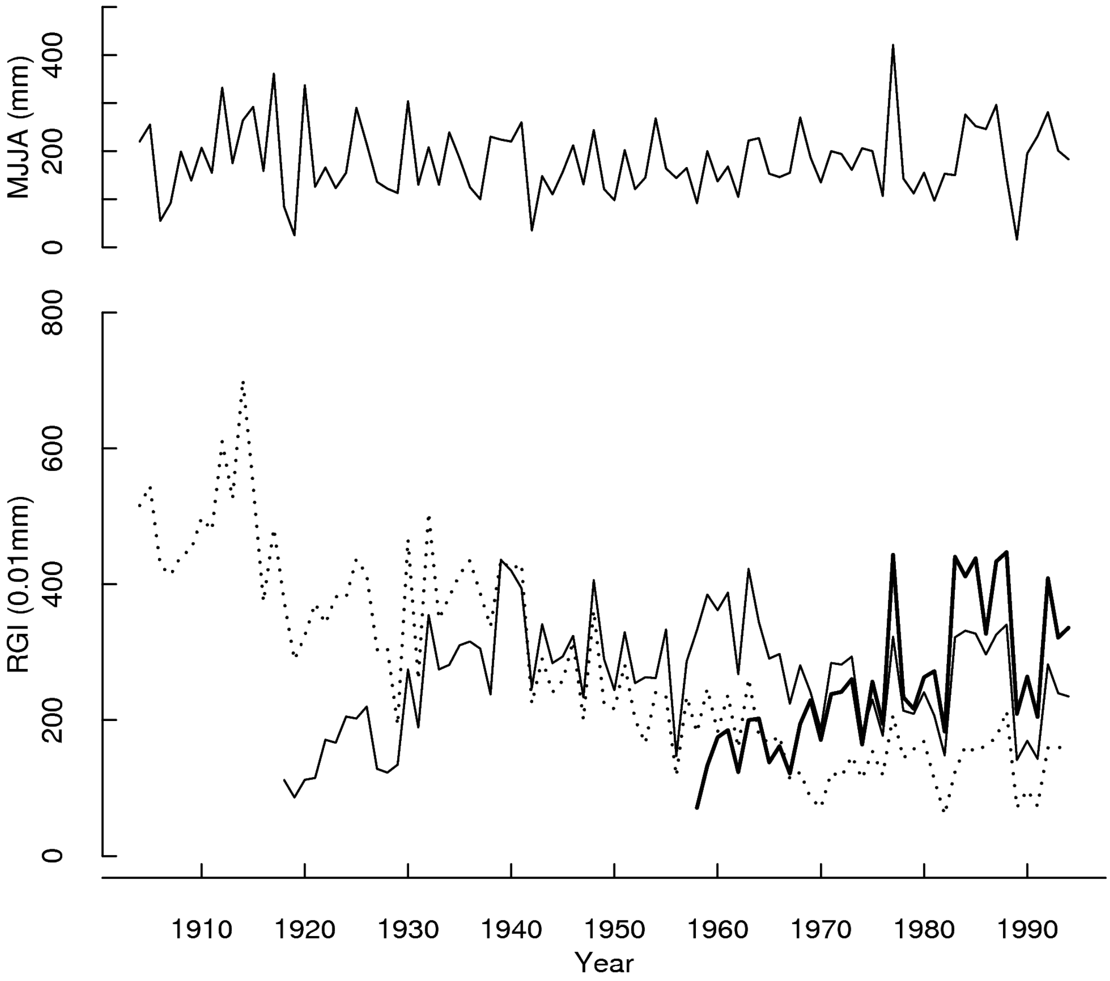

MJJA (mm)
0
200
400
RGI (0.01mm)
0
200
400
600
800
1910
1920
1930
1940
1950
1960
1970
1980
1990
Year

Figure 3

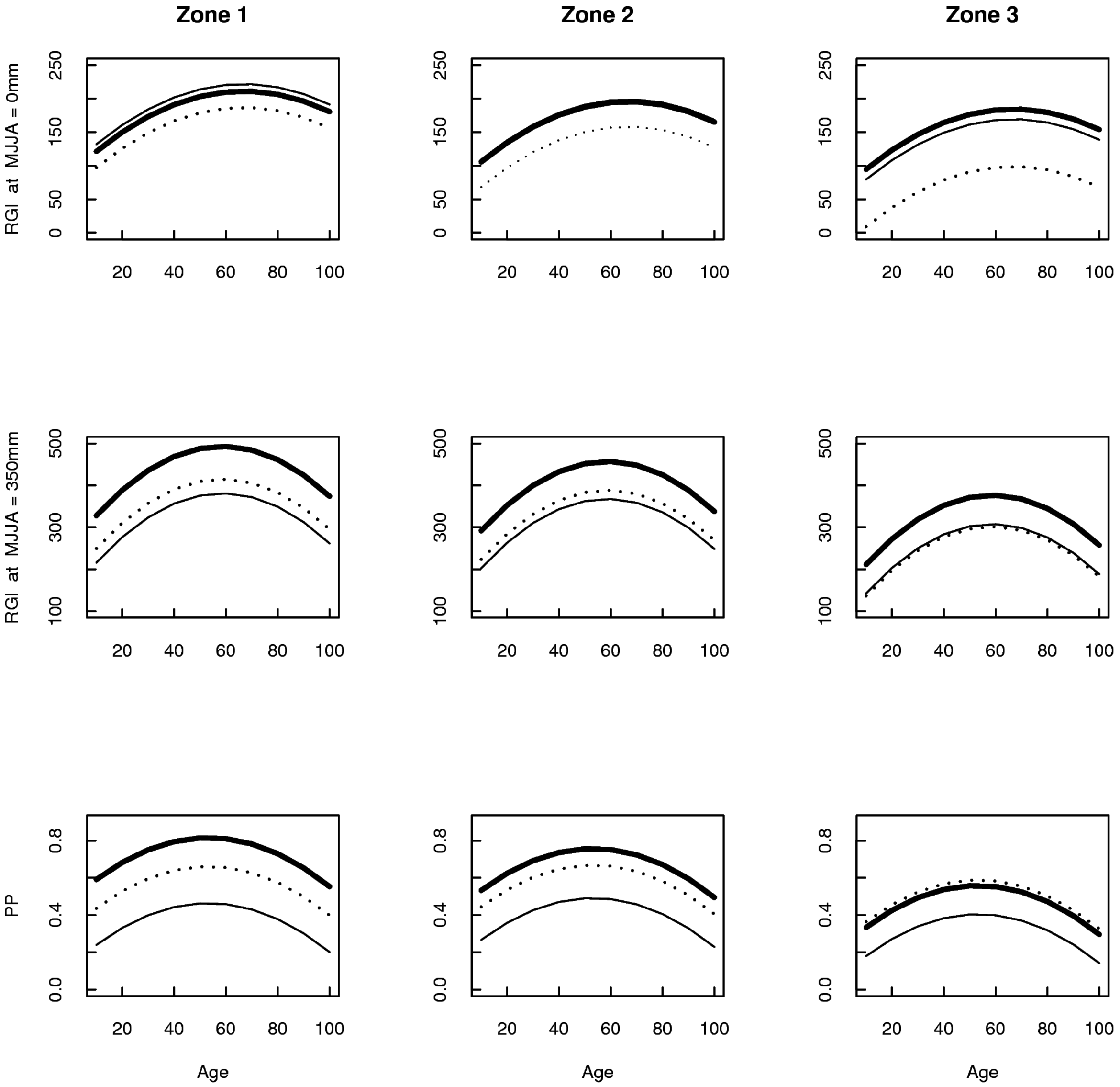
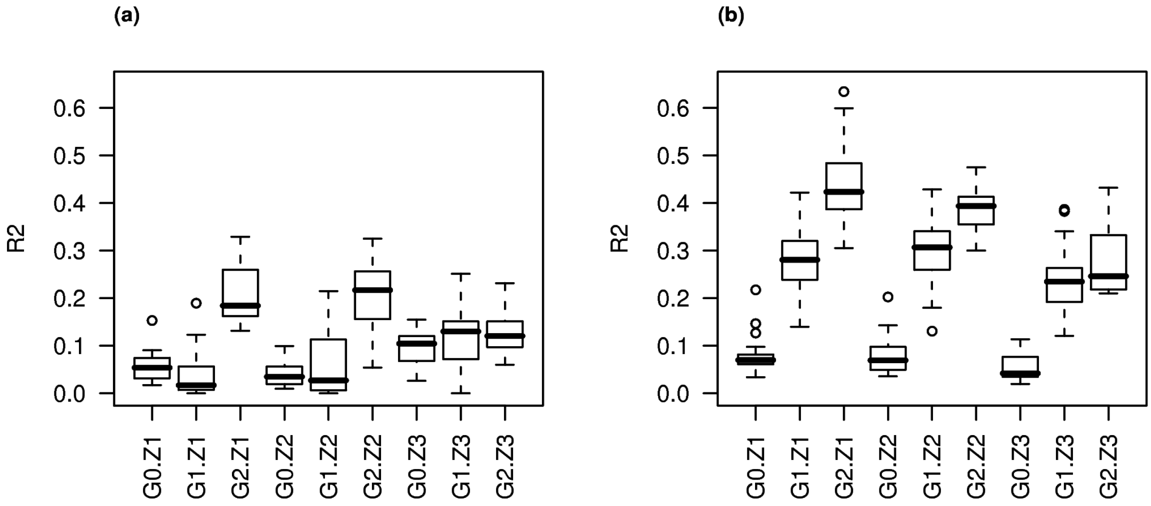

Zone 1
Zone 2
Zone 3
RGI at MJJA = 0mm
0
50
150
250
RGI at MJJA = 350mm
100
300
500
PP
0.0
0.4
0.8
20
40
60
80
100
Age
Age
Age

Figure 4



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Supplementary material

Aiming to address the points raised by the reviewers of the previous version of the manuscript, we simulated different datasets on the same experimental design as in the study and investigated the potential impact of confounding effects and complex interactions on the results of the analysis (Tables 1s, 2s and Fig. 1s). Many factors and interactions terms are included in the model and we focused on six scenarios, with a particular attention given to the fact that all generations are not observed in the same conditions (stand structure, soil texture,...) at the same age: we introduced some changes of growth and plasticity with years to check if these could induce false significant *Generation* effects. For each scenario, 20 datasets were simulated. The 120 resulting datasets were analysed using exactly the same model of analysis as in the study, in particular type III tests for fixed effects (Tables 3s, 4s), see text for further details on the model.

The main results from this simulation set were the following:

- Under the standard model investigated in the study (scenario #1), no confounding effects occured that could lead to erroneaous conclusions, except maybe the interaction *MJJA*Age* that was found significant for 3 simulated data sets over 12 successful fits.
- The evolution of the structure of the forest, which can lead to general trends of decreasing growth and/or increasing plasticity (as suggested by the reviewers), did not lead to a confounding between *Generation* and *Year* effects (scenarios #2, #3, #5, #6). In particular, the *Generation* effects (main effect and interactions) were correctly estimated despite the notably unbalanced experimental design (not all generations present each year). In scenarios #5 and #6, where *Generation*, *MJJA*Gen* and *MJJA*Zone*Gen* effects were all set to zero, the analysis did not find any of these effects as significant in a substantial number of simulations.

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- The only confounding effects found in our simulated cases were obtained when a spatial structure within zones led to significant differences among zones or generations (seven simulations with a significant *Zone* effect ; eleven with a significant *MJJA*Zone* effect ; three with significant *Zone*Gen* and *MJJA*Gen*Zone* effects ; and four with significant *MJJA*Gen* effect in scenario #4). The range simulated for the spatial effect was 50m, five times smaller than the size of the experimental plots, and smaller ranges would probably not result in such confounding. There was no significant spatial structure in the analysis of the actual dataset anyway.
- Some effects were estimated with a low statistical power considering the data structure. This is mainly the case for higher order interactions (*MJJA*Zone*Gen* and *MJJA*Zone* to a lesser extent) and for the variance-covariance parameters associated to the generations G2 (*Indiv(G2)*, *MJJA*Indiv(G2)*) and G1 to a lesser extent.
- The residual variance parameters (*Resid* and *AR(1)* parameters) were particularly well estimated in all scenarios.
- The variance of the *Individual* effect was over estimated when the true value was low (e.g. 265 to 996 instead of 100 for scenario 1); low values tend to be over-estimated for the generation G2. This confirms the low value estimated from our data set.
- The variances of the random effects were well estimated (low bias) for the slope parameter (*MJJA*Indiv* parameter) but slightly under-estimated in the scenario #4 with a spatial structure : ~ 0.03 instead of 0.04.

Table 1s: Description of the six scenarios used for simulating datasets. Scenario #1 corresponds approximately to the results obtained with the actual dataset. In the description of the other scenarios, the main changes with scenario #1 are underlined.

Scenario #1	variation among zones for growth and plasticity; no variation among generations for growth, variation for plasticity; quadratic effect of age; random variation among years; low inter-individual variance for growth and plasticity; no spatial structure of the individual effect.
Scenario #2	variation among zones for growth and plasticity; no variation among generations for growth, variation for plasticity; quadratic effect of age; growth decreases with years; low inter-individual variance for growth and plasticity; no spatial structure of the individual effect.
Scenario #3	variation among zones for growth and plasticity; variation among generations for growth and plasticity; quadratic effect of age; growth decreases with years; high inter-individual variance for growth and plasticity; no spatial structure of the individual effect.
Scenario #4	no variation among zones for growth and plasticity; no variation among generations neither for growth nor for plasticity; quadratic effect of age; growth decreases with years; very high inter-individual variance for growth and plasticity; spatial structure of the individual effect both for growth and plasticity (50m).
Scenario #5	no variation among zones for growth and plasticity; no variation among generations neither for growth nor for plasticity; quadratic effect of age; growth decreases and plasticity increases with years; high inter-individual variance for growth and plasticity; no spatial structure of the individual effect.
Scenario #6	variation among zones for growth and plasticity; no variation among generations neither for growth nor for plasticity; quadratic effect of age; growth decreases with years; high inter-individual variance for growth and plasticity; no spatial structure of the individual effect.

Table 2s: True values of the fixed effects and variance components for each scenario. See Fig 1s how the decrease in the *Year* effect was modelled in scenario #2 to #6.

	Scenario #1	Scenario #2	Scenario #3	Scenario #4	Scenario #5	Scenario #6
Fixed effects						
Intercept	150	150	150	150	150	150
Zone	0/0/-20	0/0/-20	0/0/-30	0/0/0	0/0/0	0/0/-20
Generation	0/0/0	0/0/0	0/20/40	0/0/0	0/0/0	0/0/0
*Zone*Generation*	0	0	0	0	0	0
Age	6	6	6	6	6	6
Age^2	-0.06	-0.06	-0.06	-0.06	-0.06	-0.06
MJJA	0.45	0.45	0.45	0.45	Increase	0.45
Year	Random	Decrease(1)	Decrease	Decrease	Decrease	Decrease
*MJJA*Zone*	0.25/0.15/0	0.25/0.15/0	0.25/0.15/0	0/0/0	0/0/0	0.25/0.15/0
*MJJA*Gen*	-0.15/-0.1/0	-0.15/-0.1/0	-0.15/-0.1/0	0/0/0	0/0/0	0/0/0
*MJJA*Zone*Gen*	-0.2/-0.2/0 -0.1/-0.1/0 0 / 0 / 0	-0.2/-0.2/0 -0.1/-0.1/0 0 / 0 / 0	-0.2/-0.2/0 -0.1/-0.1/0 0 / 0 / 0	0	0	0
*MJJA*Age*	0	0	0	0	0	0
*MJJA*Age*^{2}	0	0	0	0	0	0
Variance components						
Indiv (G0)	100	100	2500	4900	2500	2500
Indiv (G1)	100	100	2500	4900	2500	2500
Indiv (G2)	100	100	2500	4900	2500	2500
*MJJA*Indiv (G0)*	0.02	0.02	0.03	0.04	0.03	0.02
*MJJA*Indiv (G1)*	0.01	0.01	0.03	0.04	0.03	0.02
*MJJA*Indiv (G2)*	0.01	0.01	0.03	0.04	0.03	0.02
Cov(Indiv,PP)(G0)	0	0	0	0	0	0
Cov(Indiv,PP)(G1)	0	0	0	0	0	0
Cov(Indiv,PP)(G2)	0	0	0	0	0	0
Resid(G0)	27778	27778	27778	27778	27778	27778
Resid(G1)	27778	27778	27778	27778	27778	27778
Resid(G2)	27778	27778	27778	27778	27778	27778
AR(1)(G0)	0.8	0.8	0.8	0.8	0.8	0.8
AR(1)(G1)	0.8	0.8	0.8	0.8	0.8	0.8
AR(1)(G2)	0.8	0.8	0.8	0.8	0.8	0.8

(1) *for the MJJA*Zone*Gen interaction effects, values for Zones are in lines and for Gen in columns*

Table 3s: Number of convergent analyses and number of times each effect was found significant out of 20 simulations for each scenario. The effects that were effectively present in the simulated datasets appear in bold characters.

	Scenario #1	Scenario #2	Scenario #3	Scenario #4	Scenario #5	Scenario #6
Nb of simulations	20	20	20	20	20	20
Nb of successful fits (convergence)	12	19	20	18	17	18
Fixed effects						
Zone	**4**	**4**	**4**	7	2	**4**
Generation	0	2	1	1	1	2
*Zone*Generation*	1	1	3	3	0	0
Age	**12**	**19**	**20**	**18**	**17**	**18**
Age^{2}	**12**	**19**	**20**	**18**	**17**	**18**
MJJA	**12**	**19**	**20**	**18**	**17**	**18**
Year	**12**	**19**	**20**	**18**	**17**	**18**
*MJJA*Zone*	**10**	**16**	**16**	11	1	**18**
*MJJA*Gen*	**12**	**18**	**17**	4	1	1
*MJJA*Zone*Gen*	**3**	**8**	**8**	3	2	0
*MJJA*Age*	3	2	1	3	0	0
*MJJA*Age*^{2}	1	0	0	1	1	1
Random effects						
Indiv (G0)	0	0	**18**	**17**	**15**	**17**
Indiv (G1)	0	0	**17**	**18**	**9**	**14**
Indiv (G2)	0	2	**7**	**9**	**4**	**2**
*MJJA*Indiv (G0)*	**12**	**19**	**20**	**18**	**17**	**18**
*MJJA*Indiv (G1)*	**8**	**12**	**20**	**18**	**17**	**18**
*MJJA*Indiv (G2)*	**1**	**3**	**19**	**13**	**14**	**11**
Cov(Indiv,PP)(G0)	1	1	0	1	1	0
Cov(Indiv,PP)(G1)	1	1	0	1	2	1
Cov(Indiv,PP)(G2)	0	0	1	1	0	0
Resid(G0)	**12**	**19**	**20**	**18**	**17**	**18**
Resid(G1)	**12**	**19**	**20**	**18**	**17**	**18**
Resid(G2)	**12**	**19**	**20**	**18**	**17**	**18**
AR(1)(G0)	**12**	**19**	**20**	**18**	**17**	**18**
AR(1)(G1)	**12**	**19**	**20**	**18**	**17**	**18**
AR(1)(G2)	**12**	**19**	**20**	**18**	**17**	**18**

Table 4s: Mean estimated values of the variance components over 20 simulations for each scenario.

Var/Cov	Scenario #1	Scenario #2	Scenario #3	Scenario #4	Scenario #5	Scenario #6
Indiv (G0)	264.6	307.1	2831.5	3360.1	2161.7	2755.4
Indiv (G1)	475.8	304.5	2493.1	4745.8	2225.8	2800.9
Indiv (G2)	995.9	1372.3	3072.2	5039.3	2432.7	2133.3
*MJJA*Indiv (G0)*	0.017	0.015	0.029	0.029	0.027	0.017
*MJJA*Indiv (G1)*	0.011	0.010	0.032	0.031	0.028	0.016
*MJJA*Indiv (G2)*	0.013	0.010	0.037	0.030	0.037	0.018
Cov(Indiv,PP)(G0)	0.00	-0.10	-0.05	0.74	0.20	0.41
Cov(Indiv,PP)(G1)	-0.74	0.06	-0.31	0.09	1.01	-0.82
Cov(Indiv,PP)(G2)	-0.40	-0.23	-1.68	-0.11	0.26	0.02
Resid(G0)	26855	26761	26459	27021	26795	26186
Resid(G1)	27408	27184	27748	27741	28041	27753
Resid(G2)	28166	27353	27276	27840	27881	28318
AR(1)(G0)	0.79	0.79	0.79	0.80	0.79	0.79
AR(1)(G1)	0.80	0.80	0.80	0.80	0.80	0.80
AR(1)(G2)	0.80	0.80	0.80	0.80	0.80	0.81

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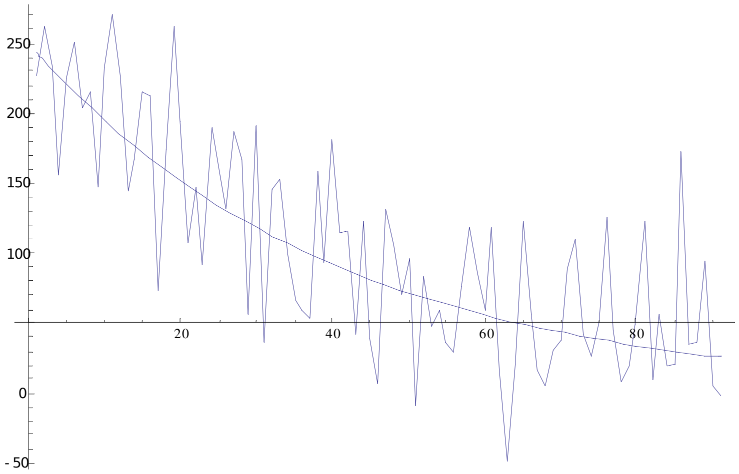
Fig 1s: Example of the decrease pattern of the *Year* effect used in scenarios #2 to #6, general trend plus random variation.



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