Université de Perpignan Via Domitia

Master Biodiversity, Ecology, Evolution

"Biodiversity and Sustainable development" Course

Part 1

Characterization of French seed sources of *Cupressus dupreziana*, the only plant species with male apomictic reproduction known to date

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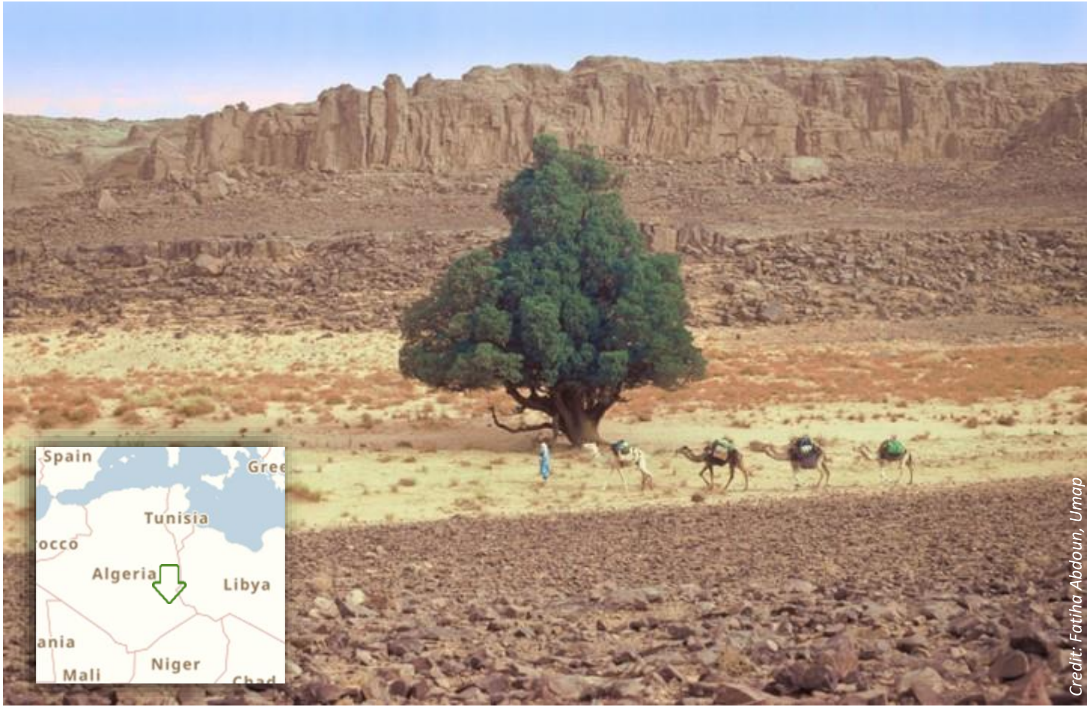

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Introduction

Tassili Cypress (*Cupressus dupreziana* A. Camus), also known as Duprez Cypress or, more locally, Tarout by the Tuaregs ("Tree of thirst" in Tamahaq), is the only conifer in the Sahara. In its natural range, it can exceed 20 m in height and 12 m in circumference. The species is strictly endemic to the Tassili N'Ajjer plateaux in Algeria, where elevations range from 1,500 to 2,000 m.a.s.l. and average annual rainfall does not exceed 30 mm (Abdoun, 2002). The presence of conifers in the middle of the desert was suspected as early as 1860, when the English explorer H. B. Tristram suggested back from an expedition that "judging from the woodwork of the Tuareg saddles, there is also a species of hard resinous wood probably related to the junipers" (Tristram, 1860). An observation that would later prove relevant insofar as the genera *Juniperus* and *Cupressus* are phylogenetically close, and both included in the Cupressaceae family.

The most recent inventory, carried out between 1997 and 2001 by Fatiha Abdoun and Mohamed Beddiaf, recorded 233 living trees, including a dozen of young individuals, unevenly distributed over a 120 km long and around 6 km wide area. Their study reveals numerous remnants of the species (trunks, branches, etc.), testifying to a population that was once much higher than the 165 dead trees recorded in 1972 (Grim, 1982). Pollen of the *Cupressus* genus discovered during sediment analyses indicates an occupation of Cupressaceae over 1,000 km from living trees, from neighbouring Libya (Mercuri et al., 1998) to northern Chad (Quezel, 1978), at a time when the humid phases of the Holocene (8,000 to 4,000 BC) formed a much richer biome than today, commonly known as the "Green Sahara".

While the oldest living specimens have been estimated to be over 2,000 years old, the desertification phenomenon affecting the region began only 500 years ago and probably forced the cypress population to persist only at high elevations, where a few seasonal streams provided an additional source of moisture to the extremely arid environment of the reg[1] and surrounding rocky slopes: "Its root system inserts itself deeply into rock crevices from which it can draw substantial moisture." (Médail, 2019). The lack of knowledge about the physiology of this cypress and the climatic conditions it faces mean that it cannot be said to be able to subsist on the frequent fogs and dews of the Tassilli plateau (Dubief, 1999), nevertheless it is accepted that most conifers are able to absorb atmospheric moisture through their foliage (Oren & Sheriff, 1995).

[1] Reg: stony desert

Its decline, estimated at 8% over a 30-year period (Abdoun & Beddiaf, 2002), has led the IUCN[2] to place the Tassili cypress on the critically endangered species red list. Natural regeneration of 2-3 trees per century is not enough to maintain the population without effective protection. Indeed, the last representatives are subject to numerous pressures, mainly linked to pastoral pruning, but also and above all to the increasing cutting of wood for bivouacs by Tuaregs, clandestine migrants and tourist camps who come to admire the rock engravings in the region (Abdoun & Beddiaf, 2002; Chad & al., 2007). The plan to protect the species drawn up in 2006 has unfortunately never come to fruition, due to a lack of political stability (Médail, 2019).

However, the Tassili Cypress's extreme resistance to drought is not the only reason for its success. Even more exceptionally, in the 2000s, a research team led by C. Pichot revealed the existence in this species of a sexual reproduction process that had never been observed in plants (Pichot et al., 2001). Normally, after meiosis, the male reproductive cell of spermaphytes has only one set of chromosomes, which, after fusion with the female reproductive cell, also haploid, gives the embryo a biparental genetic heritage. However, by observing the unusual size of pollen grains in *Cupressus dupreziana* (38 µm, the largest of all cypress species) and analyzing them by flow cytometry, the researchers discovered a high production of unreduced gametes (around 75%). By tracing the origin of this genetic singularity, an exceptional occurrence of meiotic anomalies was revealed, which in this species seem to have become the norm (El Maâtaoui & Pichot, 2001). These anomalies affect the production of healthy gametes and may explain the very low germination rate (less than 10% of seeds are normally formed). In some cases, however, they result in a diploid pollen grain, which, after penetrating the ovule, generates an embryo of exclusively paternal origin. Here, the female gametophyte is dedicated solely to the development of the embryo and its supply of nutrients. In other words, no genes are transmitted by the female way. The Tassili cypress generates clone-like embryos from the "father", developing thanks to its "surrogate mother" ability. Perhaps the most surprising consequence of this male apomixis is that *C. dupreziana* ovules also allow the development of all-paternal embryos from pollen produced by another species, *Cupressus sempervirens* L. (Pichot & al., 2008). So, depending on the pollinating species around it, a seed harvested from a Tassili cypress can just as easily give rise to a diploid Tassili cypress seedling as to a haploid and sometimes homozygous diploid *C. sempervirens* cypress by doubling the initial set of chromosomes. A few cases of interspecific triploids have also been detected. Cultivation trials carried out by INRAe between 2000 and 2005 showed that, out of 1169 cypress seedlings originated from seed-trees located in 4 ex-situ collections, the proportion of *C. dupreziana* plants varied from 30% to 50%. The others turned out to

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[2] IUCN: International Union for Conservation of Nature

be mostly homozygous *C. sempervirens* cypresses, regularly expressing strong morphological anomalies (Nava, 2008). The variability in full-seed rate and the production of *C. dupreziana* versus *C. sempervirens* embryos remain to be explained.

The present study continues the characterization of the reproductive system of Duprez cypress, by specifying in greater depth the resources that can be mobilized for this species. To this end, an experiment was set up to study seed lots and produce seedlings from a larger number of French provenances. The two objectives are to analyze i) the germination capacity of the seeds produced, and ii) the proportion of *C. dupreziana* or *C. sempervirens* embryos, depending on the origin of the stand and the surrogate mother. The aim is also to determine the stage of development of the seedlings at which confusion, on a morphometric basis, between the two species is no longer possible. This work also provided the opportunity to resume the work carried out by C. Laguerre, by updating the inventory of the species on a national scale (C. Laguerre, 2006).

Materials and Methods

Plant material

<u>Trees sampled:</u> In order to assess the variability of seed characteristics according to their origin, seeds were collected following a stratified sampling plan at 2 levels: sites and mother trees within sites. Cones were collected from October 2022 to February 2023 from 55 trees aged between 14 and 58 years, representing 12 French sites, all located in south-eastern France (Fig.1). For each of these trees, 20 cones were removed from their seeds before being weighed and measured. Seeds were stored in a cold chamber at a temperature of 5°C.

<u>Seeds and germination:</u> In total, around 135,000 seeds were collected by drying the cones before being sorted and weighed (seedlot total weight, sampling of 4*100 seeds per seedlot, determination of Weight per 1000 seeds), then immersed in a 2% dilute acetic acid bath for 90 seconds (Fig. 2a). This treatment based on the literature on seed aseptization (ITAB, 2013; Venail et al., 2017; Piyatida Inpitak and Udompijitkul, 2022) was adjusted to the size and permeability of *C. dupreziana* seeds.

The germination test was split into two series, from February 17 to May 13, 2023 for the first, and from April 14 to June 14, 2023 for the second. In each series, seeds from the same "mother" were distributed in 3 Petri dishes of 25 cm^2, thus constituting 3 repetitions. The dishes were first cleaned and then immersed for 1 hour in a 1/10° solution of 2.6% chlorine bleach. Each box contained a sheet of blotting paper sterilized by moist heat under pressure (autoclave), on which a predefined quantity of seeds was evenly distributed (Fig. 2b). Flexible forceps, pillboxes and beakers were regularly disinfected by dry heat for one hour at 140°C, and sterile gloves were used throughout seed handling.

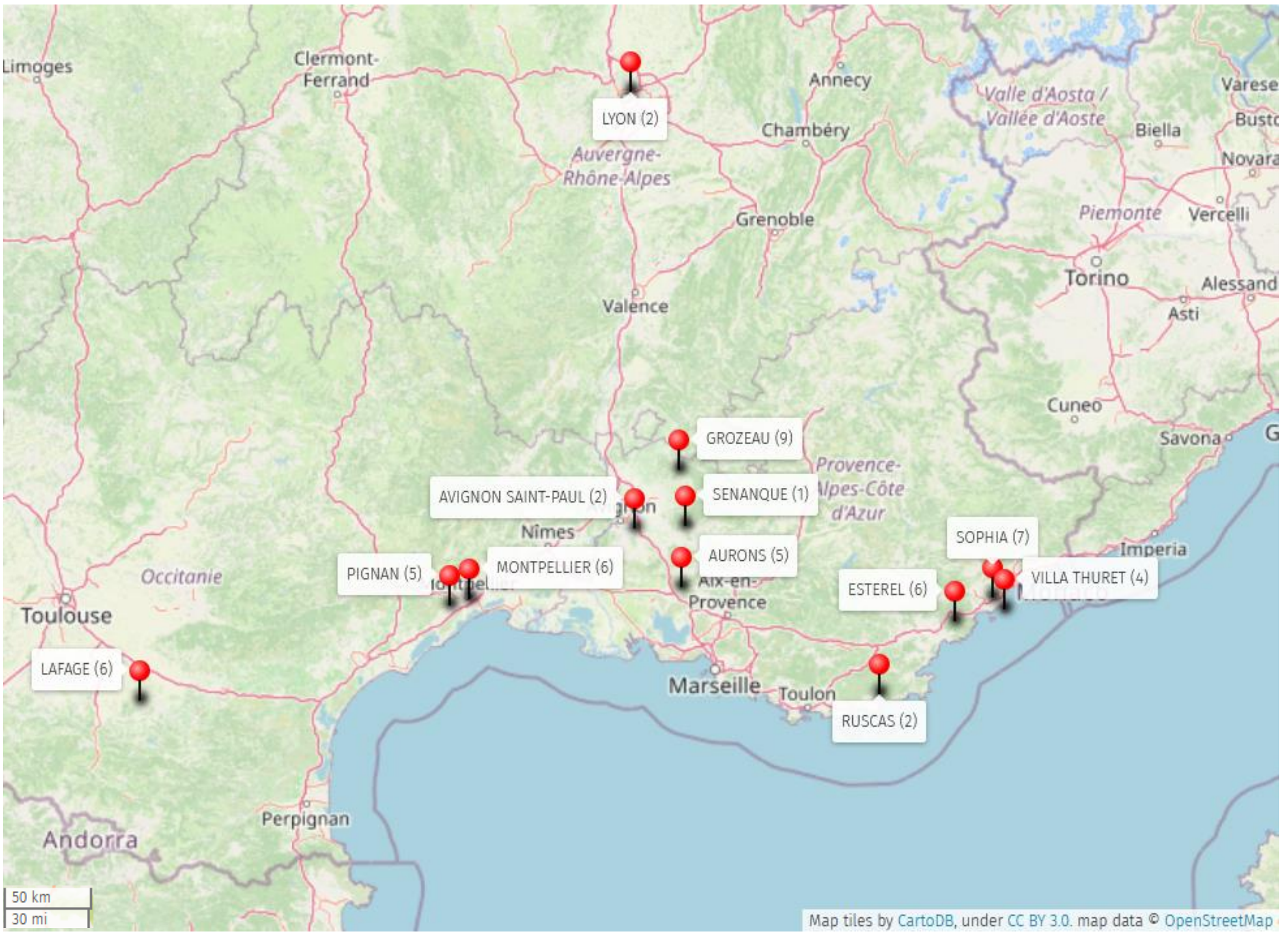


Fig. 1 Tree sites sampled (Number of cypress trees sampled)

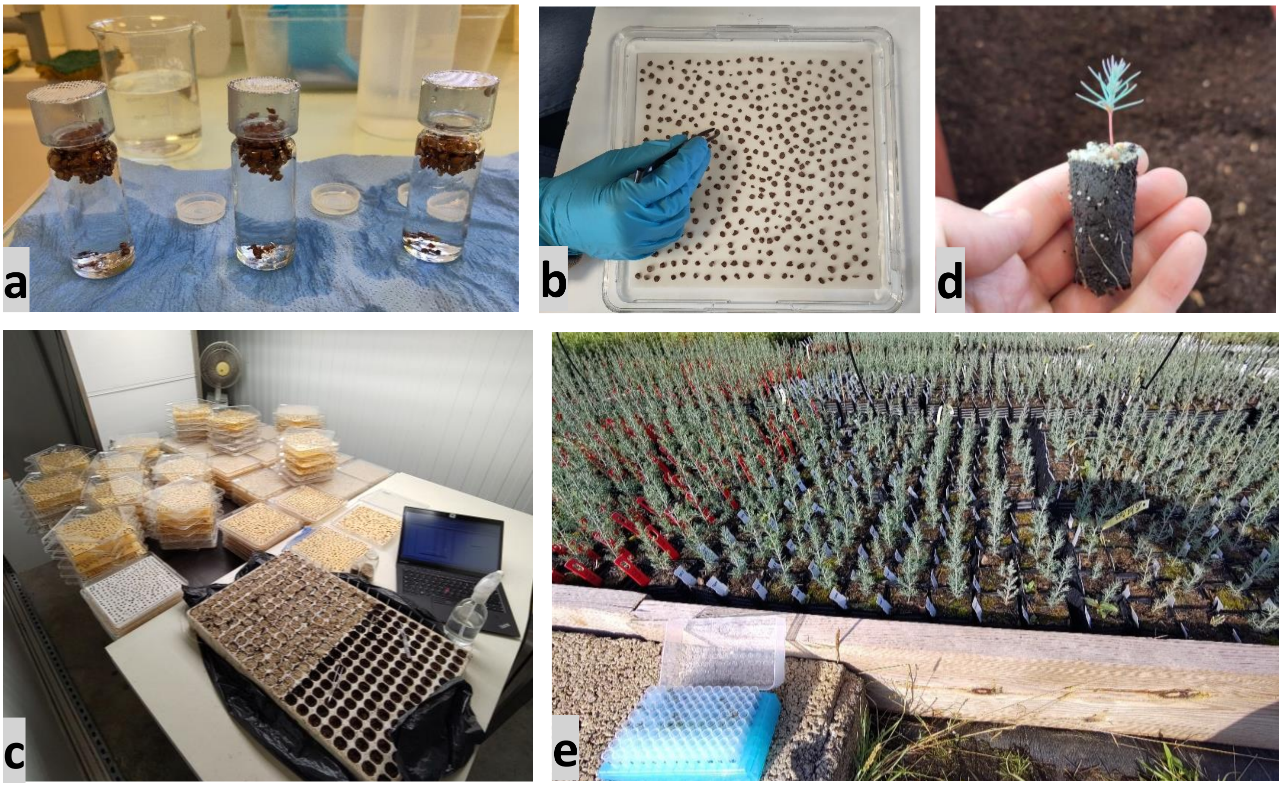


Fig. 2a-e Experimental procedure **a** Aseptization of seeds. **b** Installation of seeds in Petri dishes. **c** Sowing of seeds in soil. **d** Size of seedling during transplanting in 600cm3 containers. **e** Sampling of seedlings for genotyping.

Of the 55 seedlots included in this experiment, 10 showed clear-cut holes of around 1mm in diameter on some of their seeds, suggesting the possible presence of insect larvae in the seeds.

The 6 Petri dishes containing the seeds of a mother tree were set up in different locations: two climate-controlled chambers (Fig. 2c) (average humidity: 70.3%; avg. T°: 20.6°C; min. T°: 10.4°C; max. T°: 25.5°C; LED lighting 12h/day) and a laboratory (average humidity: 52.8%; avg. T°: 18°C; min. T°: 13.5°C; max. T°: 25.3°C; natural lighting). Humidity and temperature were recorded by HOBO Pro V2 at 5-minute intervals. The blotting papers were kept moist throughout the experiment by adding distilled water using a spray bottle. In order to characterize the seeds in terms of size and quantity, the 343 Petri dishes were photographed before germination according to a standardized protocol. The dishes were placed under a Plexiglas box surrounded by an LED strip for uniform illumination of the seeds. The photos were taken with a Fairphone 4 smartphone equipped with a 48MP sensor, positioned at the top of the Plexiglas box and leading to a resolution of 14 µm.

Regular monitoring of the germination enabled precise evaluation of the radicle emergence date, at a daily resolution. A unique number was assigned to each germinated seed, ensuring traceability throughout the experiment. The germinated seeds were transferred to germination plates comprising 285 wells, and the seedlings were raised in a culture chamber for around 2 weeks before being transferred to the Naudet nursery in Lambesc (13). The seedlings were then raised in a greenhouse for around 4 weeks, then transplanted at the appropriate time (Fig. 2d) into 600 cm^3 containers and acclimatized to outdoor conditions.

Measured characteristics

Seed size: Petri dish photographs were analyzed using scripts executed by the GRASS and QGIS software packages. These scripts enabled precise clipping of the seeds and automatic recording of the geometry and surface area of each seed in a PostgreSQL database.

Cotyledon color: Considering the bluer appearance of foliage in adult *C. dupreziana* individuals, a colorimetric analysis of cotyledons was set up to assess whether the pigment variations observed in seedlings could be used to infer species. To this end, 8 germination plates with 285 wells were photographed every 3 days for 6 to 15 days. Images were captured using a tripod-mounted Canon EOS 450D 12MP camera. The seedlings were photographed in a darkroom where no outside light could alter their hue and using the camera's flash to ensure uniform exposure for each shot (V :1/80, f :5.6). Colorimetric analysis and image processing were carried out using PostgreSQL, GRASS and QGIS softwares.

Species assignment to the seedlings

Phenotyping: We observed the morphological characteristics of the seedlings over 40 days old. Identification focused on phyllotaxis, seedling growth and leaf length and shape. A second person, a *Cupressus dupreziana* specialist, took part in this exercise on half of the plants produced, to mitigate operator bias. This collaborative approach enabled us to achieve 98% cross-validation of observations.

Genotyping: Phenotypic observations were further strengthened by a sampling approach dedicated to genotyping on around 10% of the seedlings produced. Semi-random leaf sampling was carried out throughout the production (Fig. 2e), selecting 100 seedlings identified as *C. dupreziana*, 100 seedlings identified as *C. sempervirens* and 154 seedlings for which visual identification remained uncertain. Our genetic analyses therefore involved 354 individuals, to which 23 controls were added. For this study, DNA from the samples was extracted from the leaves (50 mg) using QIAGEN DNeasy 96 plant kits, before being amplified by PCR. Genotyping was carried out on a HITACHI 3730xl DNA Analyzer capillary sequencer using microsatellite markers developed for *C. sempervirens* by Sebastiani et al. (2005) and already used by INRAe for seedlings from *C. dupreziana* mothers (Nava et al., 2009): CYP 174 (800nm), CYP 257 and CYP 258 (700nm), CYP 293 (800nm).

Environmental characterization of the sites

Meteorology: The trees sampled range in altitude from 27m (Montpellier, Hérault) to 410m (Ruscas, Var), and some of them are more than 400km apart.. As a result, it seemed appropriate to integrate the climatic conditions of each site into our analysis, focusing in particular on those associated with the pollination period of the harvested cones (January-February 2021). To do this, we used the monthly temperature and rainfall data available on the meteociel.fr website, from the weather stations closest to the sites studied (average distance: 16.7 km; maximum distance: 40 km).

Distance to the sea: To complete our analysis of environmental factors, we also included in our model the distance separating the mother trees from the Mediterranean Sea. This factor is designed to capture the effects of atmospheric humidity, marine air masses and the influence of local microclimates. These data were estimated using the uMap website.

Statistical analysis

Germination and proportion of *C. dupreziana*: We withdrew from our study the data from the 3 seedlots: THURET J2007 for the uncertain nature of the species of the mother tree and its abnormally high germination results, GROZEAU 48/303 for the low quantity of initial seeds and the total absence of germination, and LAFAGE 1, for which the seeds came from cones pollinated one year earlier than the others (2020 and not 2021).

All analyses were performed using the Rstudio statistical software. Germination rates and proportions of *C. dupreziana*, expressed as binomial data, were analyzed using generalized linear regression (GLM) with the logit link function (log(p/(1-p)) and built according to the following design: *glm(cbind(germination$Nbgermed,germination$Nbnongermed)~dateplantation+tmax01+seadist+serie+Growloc%in%serie+Prov+Mother%in%Prov, data=germination, family=binomial)*.

We assessed the effects of factors (site of origin, mother, series, growing location) and variables (seed size, distance to sea, age of mothers, temperature, and rainfall) using deviance analyses (ANOVA function with chi-square test on the GLM model).

To improve the fit with a more normal distribution, we analyzed the square root of the germination rate, which better conformed to the assumption of normality required for this statistical analysis.

Cotyledon colorimetry: We trained a model to predict species based on cotyledon color. A pre-processing phase normalized our data, taking into account the intensity level of red and green, as well as the proportions between red, green and blue (RGB). Then, the distribution of the discriminating power of the model was estimated by bootstrapping (1000 replicates with 70% of data for model calibration and 30% for model validation). For each run, the linear discriminant analysis model was used in the form: *lda(species~green+red+BGratio+BRratio+RGratio, data = train)*.

Results

Seed germination

Aseptization of the seeds and environment was very effective, despite the appearance of mycelia at the end of the process (with no impact on germination). A few larvae of Cecidomyiidae, probably the Contarinia genus that grows on cypress trees in southeastern France (Boivin & Auger-Rozenberg, 2016), were observed on around 10 seeds from the Antibes region. Around 0.5% of germinated seeds contained two embryos (Fig. 3c-d), whose natural disappearance of one of the two radicles in the more or less short term systematically left room for a single individual. Several radicles also exhibited an abnormal appearance (shape, color, truncated apex), often associated with a slower growth rate (Fig.3 e-h). As none of them survived, genetic analysis of these seedlings could not be undertaken. Nevertheless, according to Nava (2008), these morphological anomalies inherit from "genetic defects naturally present in *C. sempervirens* and here revealed due to the haploid or diploid state, probably homozygous". This inbreeding depression could explain the phenotypic aberrations observed for numerous *C. sempervirens* radicles (Fig.4 b-d) as well as the high juvenile mortality rate (25.9% of the germinating embros, most of them dying before transplanting at 6 weeks).

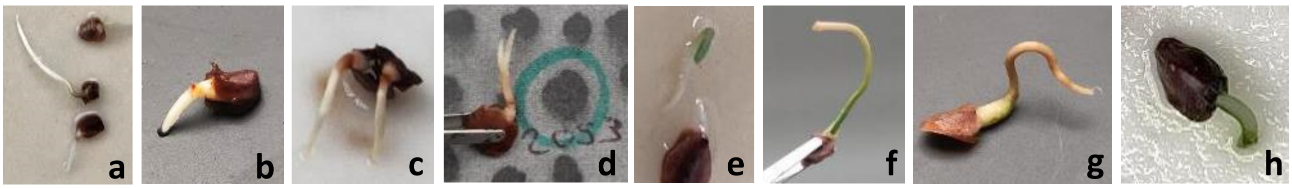


Fig. 3a-h Observation of different types of germination. **a-b** Reference radicles. **c-d** Seeds containing two embryos. **e-h** Various anomalies observed during the germination phase.

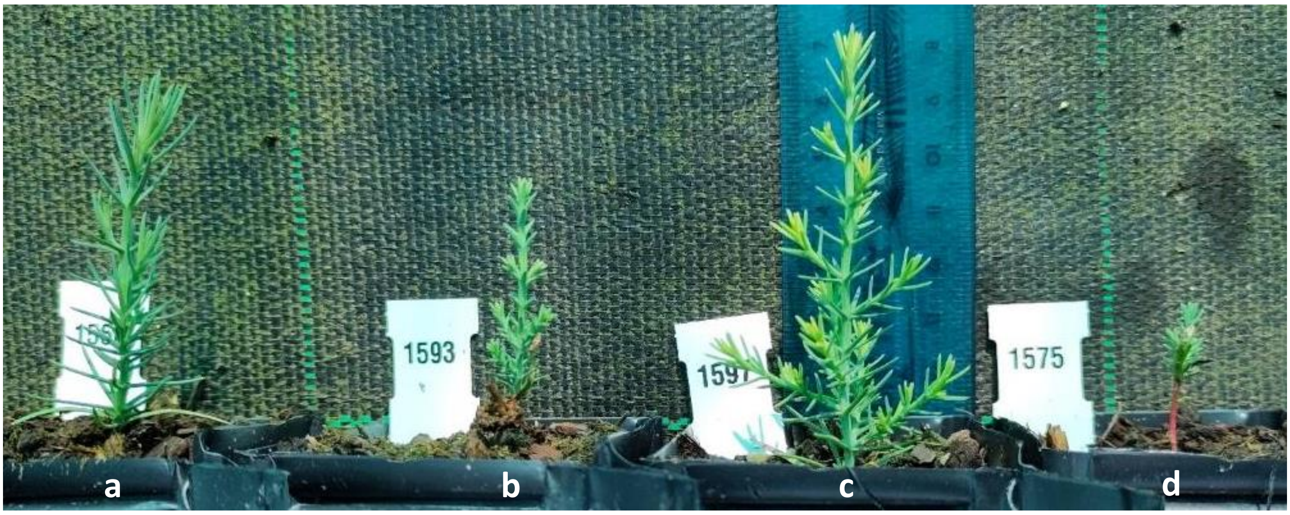


Fig. 4a-d Phenotypes of seedlings from *C. dupreziana* seeds germinated on the same day.
a *C. dupreziana* seedling. **b-d** *C. sempervirens* seedlings.

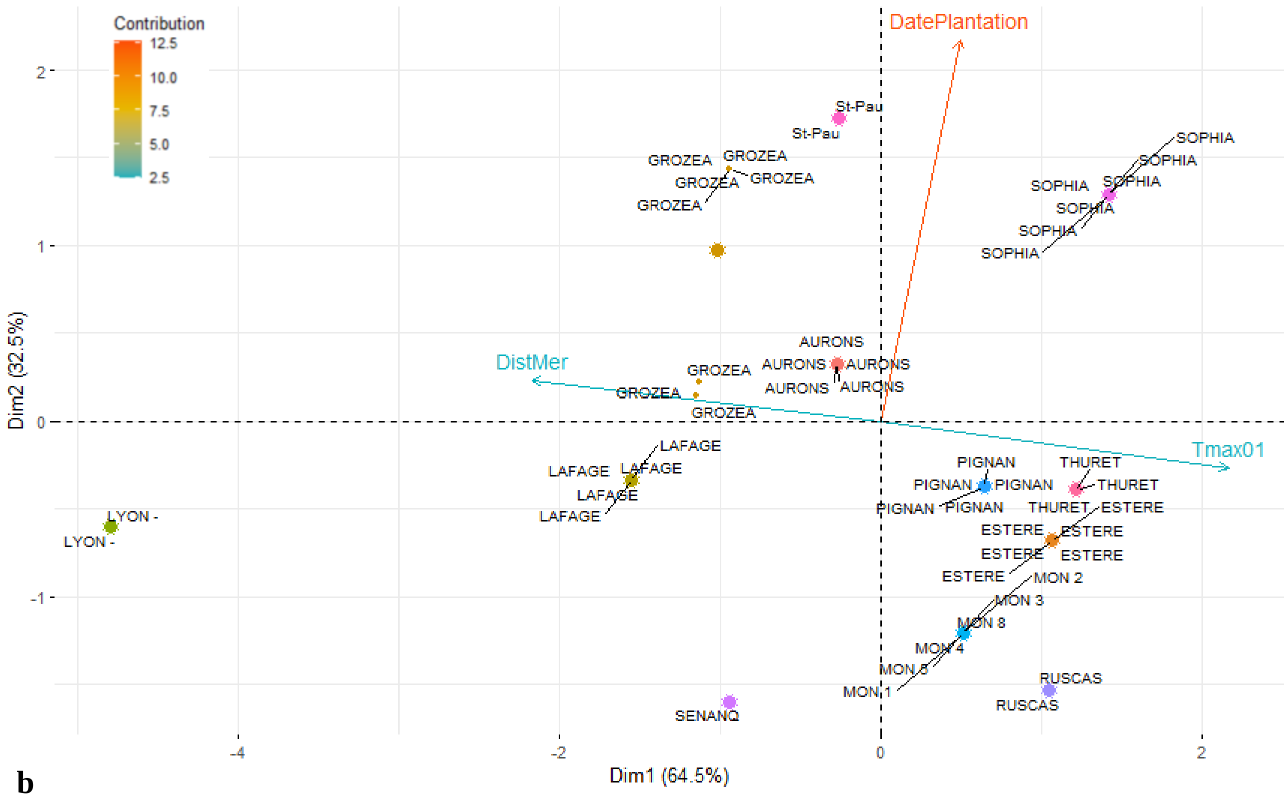
Germination analysis

The overall germination rate was 4.68% (4890 from the 104561 seeds), ranging from 0.38% to 10.99% between mothers.

All the climatic data recorded during the pollination period were highly correlated. We only retained the maximum daily average temperature of January 2021, this variable proving to be the most representative of them all (Fig.5a). The principal component analysis graph (Fig. 5b) shows the distribution of the sampled sites according to pollination weather conditions (Tmax01), distance of sites to the sea (DistMer), and the year in which the mothers were planted (DatePlantation). These include the two plantations of different ages at the Grozeau site (GROZEA), leading to distinct clusters.

<u>Analysis of variance-covariance:</u> Results (Table 1) show a strong impact of the Mother and Provenance factors, as well as a significant correlation with the maximum temperature during the pollination period and the distance of the sites to the sea. However, by reversing the order of the variables "tmax01" and "distmer", "distmer" loses its significance (Table 1a, c), suggesting that despite the strong correlation between the two variables, climatic conditions during pollination (tmax01) have a greater impact on germination than distance to the sea. We also note the limited influence of mothers' age (DatePlantation) and the absence of any effect of growing location (labo).

The significant influence of the series contrasts with that of growing location and could be linked to slightly different seed watering methods and temperature cycles.



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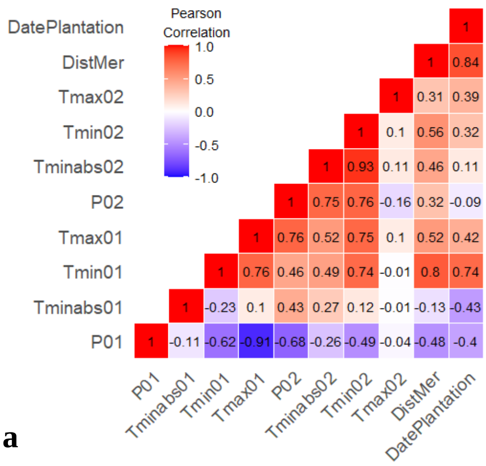

a

Fig.5 a-b Analysis of covariates
a By Pearson correlation **b** By projection (PCA)
With : **P01:** Precipitation for January 2021, **P02:** Precipitation for February 2021, **Tmax01:** Average of maximum T°C for January 2021, **Tmax02:** Average of maximum T°C for February 2021, **Tmin01:** Average of minimum T°C for January 2021, **Tmin02:** Average of minimum T°C for February 2021, **Tminabs01:** Absolute minimum T°C for January 2021, **Tminabs02:** Absolute minimum T°C for February 2021, **DistMer:** Distance to the sea, **DatePlantation:** Planting date of mother trees.

Table 1a-c Results of the analysis of variance of the effect of variables and factors on germination according to their sequence of integration in the model **(a b c).**

a

	Df	F value	Pr(>F)	
dateplantation	1	0.290	0.590578	
distmer	1	175.412	< 2e-16	***
tmax01	1	42.368	4.43e-10	***
serie	1	12.024	0.000623	***
Prov	8	36.250	< 2e-16	***
serie:labo	4	0.402	0.807042	
Prov:Mere	34	4.618	7.30e-13	***
Residuals	238			

b

	Df	F value	Pr(>F)	
tmax01	1	211.905	< 2e-16	***
distmer	1	0.427	0.513971	
dateplantation	1	5.738	0.017372	*
serie	1	12.024	0.000623	***
Prov	8	36.250	< 2e-16	***
serie:labo	4	0.402	0.807042	
Prov:Mere	34	4.618	7.3e-13	***
Residuals	238			

c

	Df	F value	Pr(>F)	
dateplantation	1	0.290	0.590578	
tmax01	1	217.653	< 2e-16	***
distmer	1	0.127	0.722253	
serie	1	12.024	0.000623	***
Prov	8	36.250	< 2e-16	***
serie:labo	4	0.402	0.807042	
Prov:Mere	34	4.618	7.3e-13	***
Residuals	238			

Generalized linear model: Applying the deviance analysis on a binomial GLM model (germinated seeds – non germinated seeds), we find results broadly similar to our previous data analysis (Table 2). On the other hand, the effect of mothers' age on germination becomes highly significant after tmax01 and distmer have fully expressed their share in the variability. The "distmer" variable remains significant whatever its position in relation to "tmax01", although the latter retains a stronger effect on germination when placed before "distmer" in the model (Table 2a, c). We should also mention the p-value associated with growing location (labo), which acquires statistical significance here despite its low value.

Table 2a-c Results of statistical analysis based on the generalized linear model evaluating the influence of variables and factors on the germination process according to their sequence of integration in the model **(a b c).**

a

	Df	Dev.Res.	Dev	Pr(>Chi)	
NULL			2282.13		
dateplantation	1	0.92	2281.21	0.337735	
distmer	1	720.54	1560.67	< 2.2e-16	***
tmax01	1	12.90	1547.76	0.000328	***
serie	1	19.61	1528.15	9.494e-06	***
Prov	8	683.82	844.33	< 2.2e-16	***
serie:labo	4	8.28	836.05	0.081767	.
Prov:Mere	34	343.28	492.77	< 2.2e-16	***

b

	Df	Dev.Res.	Dev	Pr(>Chi)	
NULL			2282.13		
tmax01	1	655.70	1626.43	< 2.2e-16	***
distmer	1	57.88	1568.54	2.780e-14	***
dateplantation	1	20.78	1547.76	5.156e-06	***
serie	1	19.61	1528.15	9.494e-06	***
Prov	8	683.82	844.33	< 2.2e-16	***
serie:labo	4	8.28	836.05	0.08177	.
Prov:Mere	34	343.28	492.77	< 2.2e-16	***

c

	Df	Dev.Res.	Dev	Pr(>Chi)	
NULL			2282.13		
dateplantation	1	0.92	2281.21	0.33773	
tmax01	1	677.44	1603.77	< 2.2e-16	***
distmer	1	56.01	1547.76	7.223e-14	***
serie	1	19.61	1528.15	9.494e-06	***
Prov	8	683.82	844.33	< 2.2e-16	***
serie:labo	4	8.28	836.05	0.08177	.
Prov:Mere	34	343.28	492.77	< 2.2e-16	***

Analysis of *C. dupreziana* proportion in seedlings

Due to the loss of identity for some seedlings, the dataset was reduced to 4631 individuals. Based on Nava's work, dead individuals were assigned to the *C. sempervirens*.

Analysis of covariance: Using the same methodology as for germination rate, we tested the individual impact of covariates on *C. dupreziana* rate by modifying their order of integration in the model. The results (Table 3) show a very strong effect of the age of the mothers and a significant influence of temperature during pollination (tmax01), a variable highly correlated with distance to the sea. However, the latter (distmer) has less impact on the proportion of *C. dupreziana* than Tmax01, probably due to its lesser consideration of the species compared with the Tmax01 variable.

Table 3a-c Results of the analysis of variance of the effect of variables and factors on the rate of *C. dupreziana* according to their sequence of integration in the model (**a b c**).

a

	Df	F value	Pr(>F)	
dateplantation	1	41.381	7.33e-10	***
distmer	1	30.928	7.50e-08	***
tmax01	1	13.928	0.00024	***
serie	1	0.755	0.38581	
Prov	8	13.270	1.08e-15	***
serie:labo	4	0.722	0.57767	
Prov:Mere	34	3.530	7.69e-09	***
Residuals	227			

b

	Df	F value	Pr(>F)	
tmax01	1	27.912	2.97e-07	***
distmer	1	4.700	0.0312	*
dateplantation	1	53.625	4.19e-12	***
serie	1	0.755	0.3858	
Prov	8	13.270	1.08e-15	***
serie:labo	4	0.722	0.5777	
Prov:Mere	34	3.530	7.69e-09	***
Residuals	227			

c

	Df	F value	Pr(>F)	
dateplantation	1	41.381	7.33e-10	***
tmax01	1	43.727	2.67e-10	***
distmer	1	1.128	0.289	
serie	1	0.755	0.386	
Prov	8	13.270	1.08e-15	***
serie:labo	4	0.722	0.578	
Prov:Mere	34	3.530	7.69e-09	***
Residuals	227			

Generalized linear model: As expected, the results obtained with GLM (Table 4) do not fundamentally change the conclusions obtained with ANOVA. Planting date and temperature at pollination remain the variables with the greatest influence on the rate of *C. dupreziana*, and there is a strong correlation between tmax01 and distmer. We observe that the influence of the series effect takes on a considerable magnitude, which we attribute to the increased complexity of the identification of seedlings in series 2 due to their youth, and to the fact that this identification was carried out by a single observer.

Table 4a-c Results of statistical analysis based on the generalized linear model evaluating the influence of variables and factors on the rate of *C. dupreziana* according to their sequence of integration in the model **(a b c).**

a

	Df	Dev.Res.	Dev	Pr(>Chi)	
NULL			855.14		
dateplantation	1	62.353	792.79	2.871e-15	***
distmer	1	38.750	754.04	4.817e-10	***
tmax01	1	8.296	745.74	0.003973	**
serie	1	32.175	713.56	1.409e-08	***
Prov	8	267.164	446.40	< 2.2e-16	***
serie:labo	4	5.658	440.74	0.226165	
Prov:Mere	34	150.996	289.75	< 2.2e-16	***

b

	Df	Dev.Res.	Dev	Pr(>Chi)	
NULL		277	855.14		
tmax01	1	276	838.86	5.468e-05	***
distmer	1	275	838.52	0.5589	
dateplantation	1	274	745.74	< 2.2e-16	***
serie	1	273	713.56	1.409e-08	***
Prov	8	265	446.40	< 2.2e-16	***
serie:labo	4	261	440.74	0.2262	
Prov:Mere	34	227	289.75	< 2.2e-16	***

c

	Df	Dev.Res.	Dev	Pr(>Chi)	
NULL			855.14		
dateplantation	1	62.353	792.79	2.871e-15	***
tmax01	1	46.929	745.86	7.361e-12	***
distmer	1	0.118	745.74	0.7315	
serie	1	32.175	713.56	1.409e-08	***
Prov	8	267.164	446.40	< 2.2e-16	***
serie:labo	4	5.658	440.74	0.2262	
Prov:Mere	34	150.996	289.75	< 2.2e-16	***

Prediction of germination and species ratio within seed lots

The generalized linear model is not only better suited to non-normed data and different sample sizes than analysis of variance, but in its binary form (sprouted seeds - unsprouted seeds; *C. dupreziana* - *C. sempervirens*) it also enables more accurate predictions of the estimated values, using a link function to relate the independent variables to the probability of success (germinated seed or *C. dupreziana*). Reliable confidence intervals of the germination rates and *C. dupreziana* proportions were estimated in the estimation space of the binomial model, before reprojecting them in the initial space by the inverse function (exp(x)/(1+exp(x)).

The estimates show the clear segregation of germination rates attributable to mother trees and their provenance (Fig. 6). The contrast is particularly visible: on the one hand, at the "Grozeau" site (northern Vaucluse), where germination rates are moderate but stable and relatively uniform, and on the other hand at the "Pignan" site (south-east of Montpellier), where despite a more pronounced "mother" effect, the overall germination rate is the highest (9.4%).

With regard to the proportion of C. dupreziana, the site clusters are less marked than for germination, but nevertheless very distincts (Fig. 7). Mothers sampled in the Esterel massif and at Pignan show by far the lowest variability, but also the highest overall proportions of C. dupreziana, with average rates of 76.2% (from 71.9% to 80.8%) and 72.8% (from 64.7% to 79.5%) respectively. The Villa Thuret site (3 seed_trees) comes close to these values (71.9%). It is interesting to note that all 3 sites are less than 10 km from the sea, and therefore benefit from a particular climate. Moreover, the trees on these

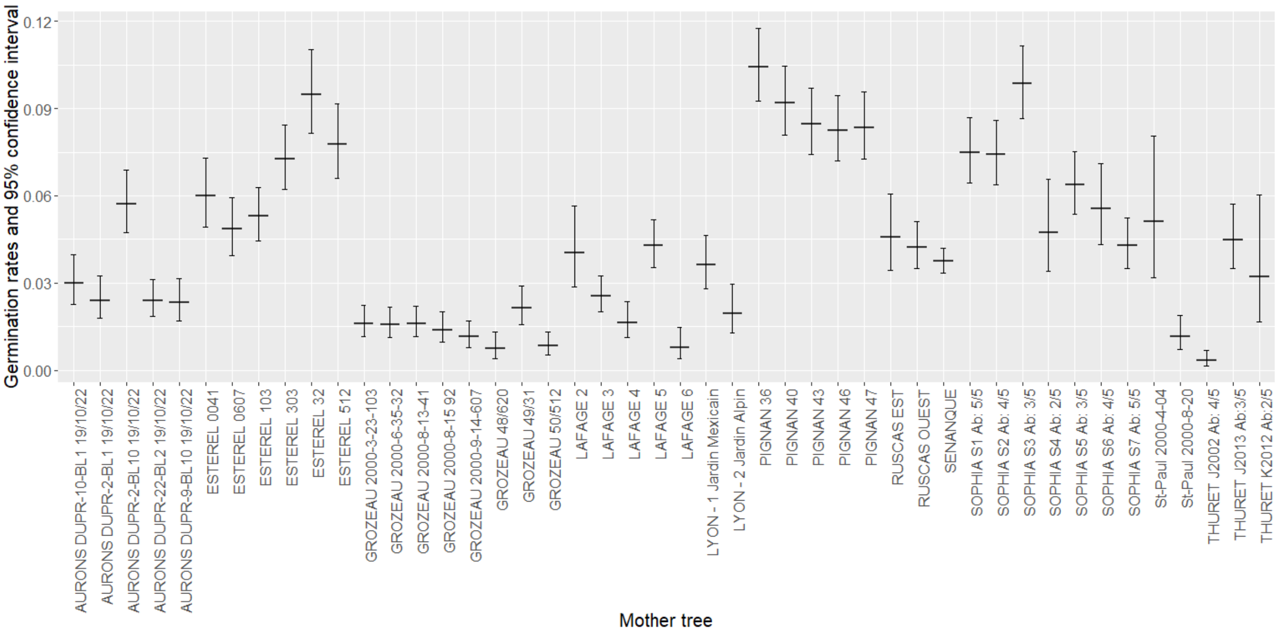

Fig. 6 Estimation of germination rates by "Mother" with 95% confidence intervals

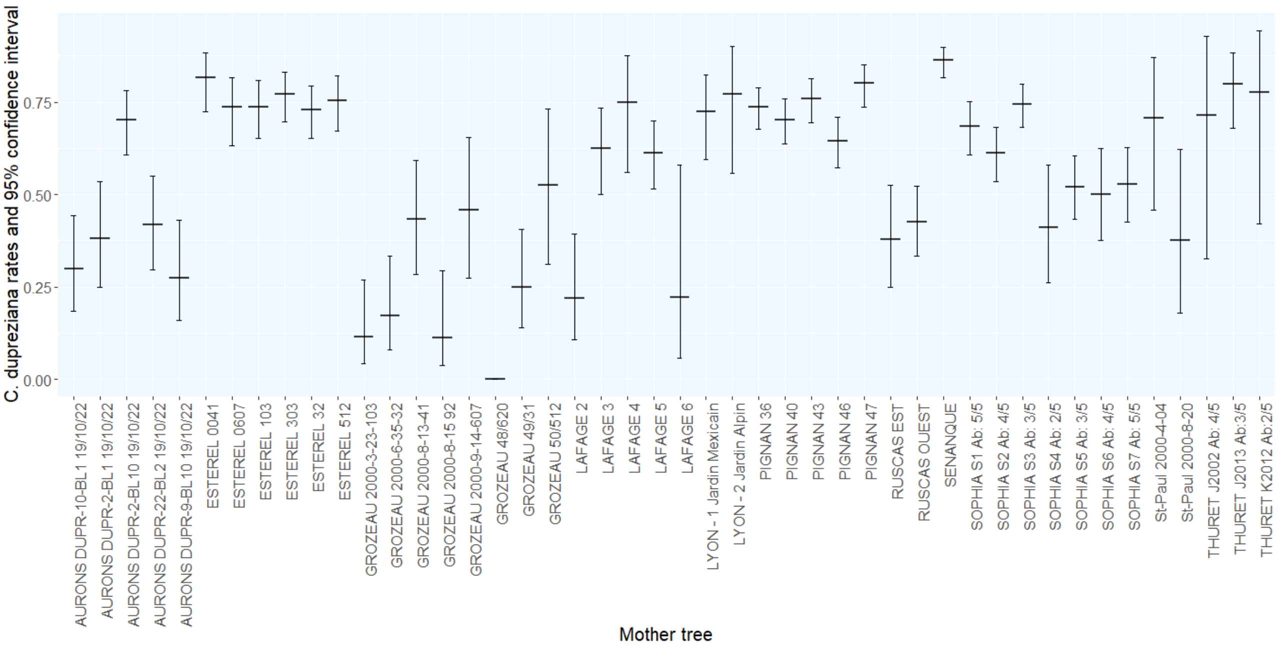

Fig. 7 Estimated rates of *C. dupreziana* produced by "Mother" with 95% confidence intervals

sites are among the oldest (Esterel: 42 years, Pignan: 39 years VT: 38 years). Age effect is particularly notable for Sénanque, where the oldest mother (58 years) produces the highest proportion of *C. dupreziana* (86%). Conversely, the only mother to have produced only *C. sempervirens* seedlings is at the Grozeau site (age: 36 years, distance to sea: 100 km), although the results for the latter are not very representative (only 4 germinated seeds). Overall, the graphs in figures 6 and

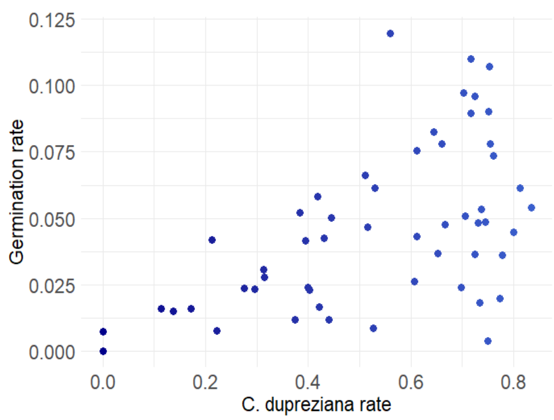

Fig. 8 Correlation between germination and seedling rates of *C. dupreziana* (per "mother")

7 indicate a positive correlation between germination rates and *C. dupreziana*, which was confirmed by a correlation coefficient of 0,46. However, this correlation is not linear but "triangular" (Fig. 8), demonstrating that high germination rates are always associated with high proportions of *C. dupreziana*.

Seed/seedling characterization by species: The stage of development allowing species identification was assessed on seed and seedlings characteristics. Although slightly correlated to species, seed size (estimated for 2167 seeds) cannot be efficiently used for this purpose.

The colorimetric analysis of cotyledons was realized through a linear discriminant analysis on 2 subgroups, one encompassing all the photos (Group 1 - 339 cotyledons), the other focusing solely on the last seedling stage before transfer to the nursery (Group 2 - 130 cotyledons). The linear combination of color and color ratios used as discriminant variables led to little distinction between species, with cotyledon colors tending to harmonize with seedling age. Difference was mainly due to the green value, higher in *C. dupreziana*. Based on 1000 bootstrap estimates, the probability of correct attribution to a species was around 0.7, ranging from 0.6 to 0.8.

Discussion

The results of our analyses, carried out on over 100,000 seeds, show that seed germination rates and proportion of *C. dupreziana* versus *C. sempervirens* embryos are very significantly correlated with the mother trees and their provenance. The differences observed between them are probably linked, not to their genome, but to their conditions of exposure to pollen. In the case of germination rates, the variability between sites depends mainly on the climate at the time of pollination, whereas *C. dupreziana* rates seem to be more influenced by the age of the mothers. In this respect, it is interesting to note that the Esterel massif provenance, one of the largest and oldest concentrations of Tassili cypress in France, had an overall germination rate in 2001 ranging from 0.22% to 3.9%, with an average *C. dupreziana* rate of 23% (Nava, 2008). In 2023, germination rates for the same provenance range from 2.3% to 11.9%, with an average proportion of *C. dupreziana* reaching 76.2%. It is highly probable that tree maturation may explain this phenomenon. The increase of pollen production with age leads to greater pollination of individuals, and therefore higher germination and above all *C. dupreziana* rates. With a view to mass reproduction of the species, this would suggest directing seed harvesting to the oldest trees and, if possible, checking that they are well pollinated (from mid-January to mid-February) 1.5 years before harvest, by observing the color of the tree foliage when male cones located at the end of the short twigs turns to yellow.

With regard to the phenotypes of *C. dupreziana*, and still with a view to large-scale seedling production, it seems difficult to identify with certainty the species of seedlings before they have reached their first month, although more or less pronounced morphological anomalies in seedlings remain a good indicator of *C. sempervirens*. We thought it would be interesting to pursue this research using near-infrared spectroscopy (NIRS). This rapid, easy-to-use technique is now widely used in the agricultural industry to determine the internal structure of seeds and ensure they are free of defects (Pansare et al., 2012; Nicolaï et al., 2014). We initiated studies in this direction, the conclusions of which are still to come.

Studying and propagating the Tassili Cypress has at least three major benefits. Firstly, to preserve in ex-situ conditions a species on the verge of extinction in its native range. Although its exceptional characteristics have enabled it to survive the centuries in extreme conditions, its disappearance from the desert is now a serious concern. On the other hand, its integration into French forests, and other Mediterranean locations, would offer a promising prospect of evolving into ecosystems better adapted to future environmental conditions. In the absence of any adaptive evolution, however, this cypress would have to be planted sporadically, as a companion species or as an ornamental. The establishment of monospecific stands over large areas could present risks, particularly in the event of parasitic attack. Finally, its unique "surrogate mother" strategy opens up a vast field of research into gymnosperms, their genetic variability and the expression of recessive genes. The production of haploid lines is already being used to select non-pollinating ornamental varieties to prevent allergy to Cupressaceae pollen. These lines are also being used as part of the sequencing of the green cypress genome (INRAe, 2023) and could eventually be extended to the production of varieties selected for their rapid growth or resistance to pathogens.

C. Laguerre (2006) pointed out that France was home to four times more *C. dupreziana* specimens than the Tassili n'Ajjer plateau. An update of her inventory brought this number to seven, with over 1,500 trees, most of them located in the south-east of France (Appendix 1). If we consider that the survival of this species seems to be closely dependent on human action in its favor, this increase is a source of encouragement, all the more so as it does not take into account the 3139 individuals from our production and presently in nursery. We regret, however, that these trees are not fully representative of the relict population. The updated inventory and the work of L. Nava indicate that most of the seeds used in ex-situ collections come from Tamrit, the most easily accessible region of the natural range. Although this species has a quasi-unique genome, variants have been detected in samples from the north of the natural area (Nava, 2008). Knowledge of its genetic heritage is lacking, and it would be essential to remedy this by mobilizing all the resources of the natural area, which could easily be achieved by collecting seeds or grafts from the 233 trees still alive.

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Appendix 1

National inventory

From 2006 to 2008, Laguerre and Nava (Nava, 2008) drew up a fairly exhaustive inventory of French Tassili Cypress resources, identifying 1,026 trees throughout mainland France. In order to monitor the evolution of this resource, it now seemed appropriate to update the inventory by checking the persistence of known trees and adding new individuals. To do this, a survey was carried out among those already identified and extended to over 190 arboretums, as well as the main public bodies, associations and private collectors. A file was created containing all the information relating to the trees inventoried, including their altitude, age, the substrate on which they grow, as well as the origin of the information and the contact details of the owners.

The census revealed the disappearance of around fifty trees, particularly in the Carpentras and Montfavet communes. However, plantings by several private owners, arboretums and the integration of the former FCBA[3] experimental plots have increased the overall number of representatives of the species in France, which we were able to estimate at 1,534 individuals. Figure 9 illustrates their distribution, with the sites used as a basis for this study highlighted in red.

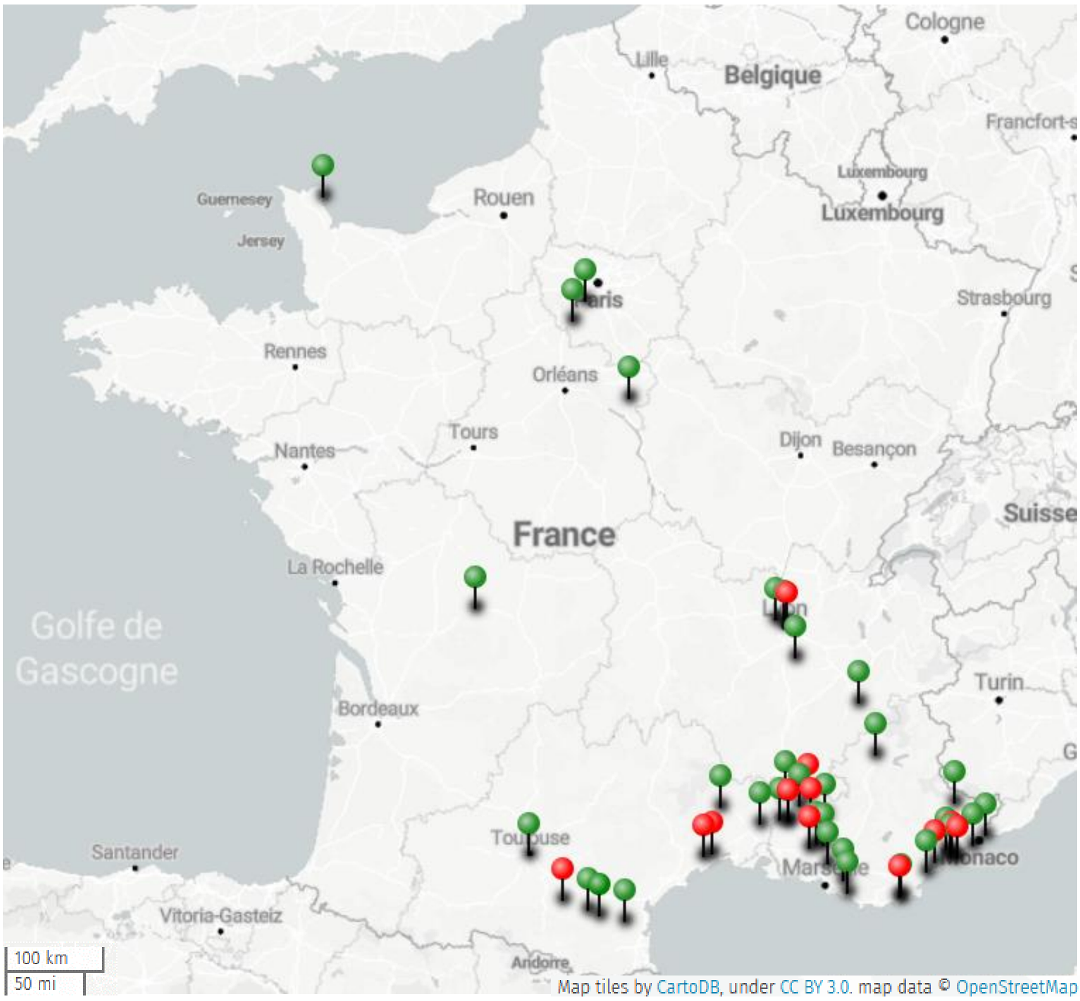

Fig. 9 Distribution of French *C. dupreziana* resources (sampled sites in red)

[3] FCBA : Technological Institute Forest Cellulose Wood Construction Furniture

Abstract

The study of the adaptive and genetic characteristics of endangered species is an essential component to define their conservation strategy. Among the most endangered, the Tassili cypress (*Cupressus dupreziana* A. Camus), native to the Tassili N'Ajjer plateau in Algeria, now numbers just 233 individuals in its natural range. Its male apomictic mode of reproduction works on the surrogate mother principle and also produces under ex-situ conditions *C. sempervirens* embryos. Our study focuses on characterizing french resources, for their germinative capacity and the proportion of individuals of each species. Species determination is based on morphological criteria and, for a sub-sample, on microsatellite markers. The average germination rate was 4.7% and 2/3 of seedlings were *C. dupreziana*. Germination and proportion of *C. dupreziana* vary greatly according to provenance and mother tree. The germination rate is highly correlated with the temperature of the month of pollination, while the proportion of *C. dupreziana* is more closely explained by the age of the mother trees, as older trees produce more pollen. Distinction between species remains difficult until the seedlings are a month old.

Phylica arborea is another worrying example of an endangered species. This tree is found only on the islands of Amsterdam and Tristan da Cunha, 7,000km apart and among the most isolated in the world. On Amsterdam Island, in the heart of the Indian Ocean, its population has plummeted by 99% in three centuries as a result of multiple anthropogenic pressures. The bottleneck suffered by the species may have greatly reduced its resilience in the face of hazards. We propose management measures to safeguard the species based on genome sequencing, assessment of its adaptive potential and, if necessary, integration of resources from Tristan da Cunha.

Keywords: Conservation, endangered species, genetic diversity, apomixis, adaptive potential, germination, genetic resources