B4EST

Adaptive BREEDING for productive, sustainable and resilient FORESTs under climate change

Deliverable D4.7

Guidelines for the optimization of genetic gains and seed production in maritime pine seed orchard

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1 Summary

Improved maritime pine seed production is facing major difficulties such as invasive pests or climate change leading to a decrease in seed production, or such as pollen contamination from surrounding non-improved stands having a negative impact on the genetic value of the produced seeds. In B4EST project, we are testing on a small scale a greenhouse seed orchard, to improve the quality and quantity of seed production. This deliverable is reporting on performance of an experimental greenhouse seed orchard and guidelines for establishment of new seed orchards limiting pollen contamination and abiotic/biotic impacts on seed production. This experiment confirms the low seed yield observed in seed orchards, even though pollinated flowers and conelets were protected with bags suggesting multifactorial causes of cone abortion and empty seeds. Researches related to the drop in seed production due to biotic and abiotic stresses must be strengthened in order to make recommendations for seed orchard managers. This experiment also confirms efficiency of mass pollination in greenhouse conditions to limit pollen contamination when commercial seed orchards are established in an area where maritime pine is dominating forest composition. Further needs will also concern evaluation of cost of establishment and management of greenhouse seed orchard for maritime pine as this experiment was realized at a very limited scale with young grafted trees.

2 Background

Tree breeding programs aim at producing improved plant material to use for afforestation. The best performing trees for adaptation, productivity or resistance to pest or pathogens, are selected to establish seed orchards managed to produce seeds by inter crossing. Harvested seeds are then used to produce seedlings for afforestation. In this way, the genetic gain provided by the breeding program is deployed in the forest to better provide wood and services. Maritime pine (*Pinus pinaster*) is the first planted species in France with between 25 to 45 million seedlings sold per year (Figure 1). The breeding program developed through GIS pin maritime du future has led to several generations of seed orchards since its start around 1950. Currently, seeds are collected on a third generation of seed-orchards called VF3 seed orchards planted between 2002 and 2006 (190 ha) but establishment of a new seed orchard generation, VF4, is in progress. Two types of seed orchards are considered for maritime pine: open-pollinated clonal seed orchards and polycross seed orchards (Baradat 1987). These seed orchards are extensively managed by private and public operators.

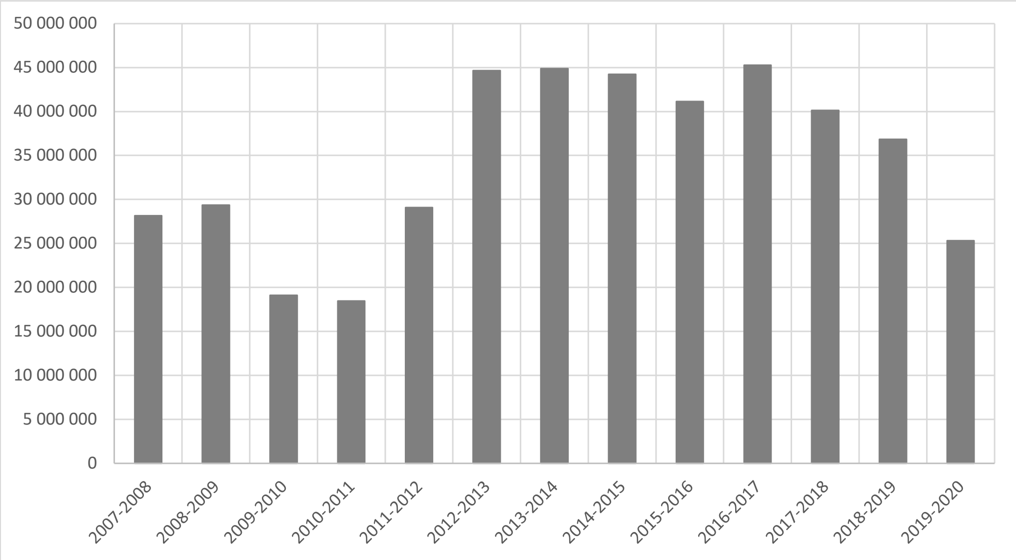

Figure 1. ***Maritime pine seedlings sold in France from 2007 to 2020*** (data compiled from https://agriculture.gouv.fr/statistiques-annuelles-sur-les-ventes-de-graines-et-plants-forestiers)

The conditions of seed production are currently changing. Invasive pests (*Leptoglossus occidentalis*) attacks probably combined with climate change impacts (summer drought, spring frost) are drastically decreasing seed yields. Collected seeds on the VF3 seed orchards decreased from 3 000 kg in 2016 to 500 kg in 2020. As the VF3 seed orchards were established from 2002 to 2006, a production increase after 2016 was expected in the absence of new biotic or abiotic stresses (seed orchard area was sized based on a minimum seed production of 15 kg/ha). The seed production drop observed after 2016 (Figure 2) is due to the combination of **cone production decrease** (number of cones per tree) and **seed yield decrease** (number of seeds per cone). Studies are in progress to better identify the different causes of this seed production drop. Physical protection around female flowers and cones on controlled crosses of the breeding program increased significantly seed production suggesting the major role of insects. Various chemical treatments applied by micro-injection in the stem or by spraying on the cones in spring and summer are currently evaluated. First results with emamectin benzoate by micro-injection seem promising (Puiseux and Mercadal 2021). However, the low seed yield encountered in recent years already forces orchard managers to harvest former generation seed orchards to reconstitute their stocks. Seed production is consider as a limiting factor of genetic gain deployment and solution to maintain or increase it is one important priority for maritime pine but also for other forest tree species of interest.

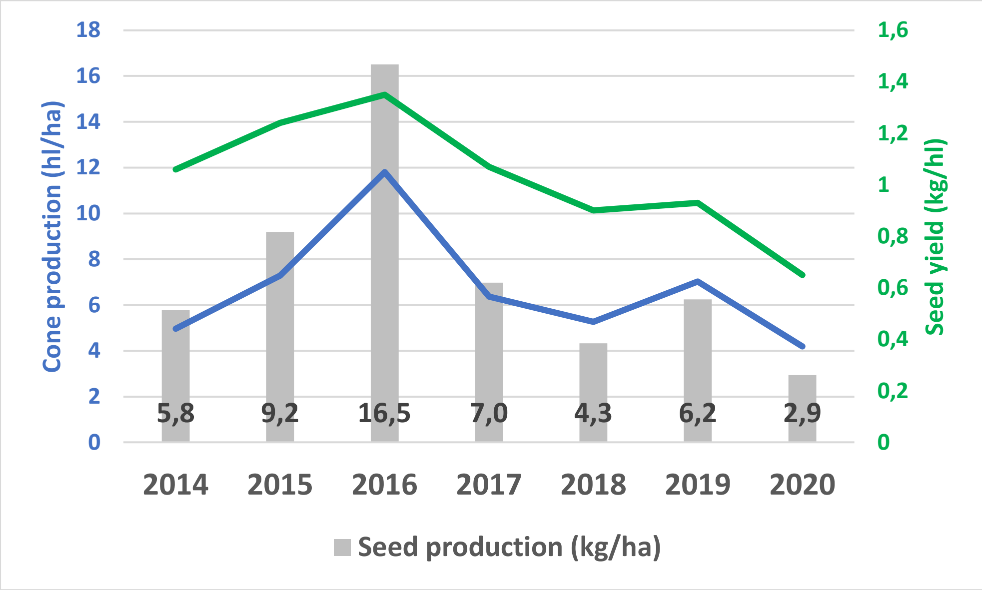

Figure 2. ***Annual maritime pine seed production per hectare from 2014 to 2020*** in VF3 seed orchards [adapted from Puiseux and Mercadal (2021)]

The extensive management of maritime pine seed orchards, in particular open-pollination, raises the risk of **pollen contamination** from surroundings stands. This has effectively been observed and estimated with molecular markers: Plomion et al. (2001) have reported a pollen contamination rate of 36% using chloroplast DNA microsatellites and even more (32% to 81%) using nuclear microsatellites (Plomion et al. 2005). More recently, a large study (Bouffier et al. 2017) has been carried out with SNP markers on more than 2,500 seeds collected in three VF3 seed orchards at successive years. This study reveals a mean pollen contamination rate of 50% (Figure 3). VF3 seedlots are supposed to deliver 40% genetic gains for volume and stem straightness in comparison to unimproved material. Assuming that foreign pollen comes from unimproved stands, expected genetic gains drop from 40% to 30%. It was also highlighted that pollen contamination can be drastically limited: 1/ by choosing carefully the location of the orchard based on the distance from external pollen sources and on the soil nature allowing earlier (or delayed) blooming in seed orchards, 2/ by avoiding seed collection at young age. However, some yearly variations in pollen contamination were observed and could be associated with reduced rainfall during pollination period.

In the literature, various management practices have been suggested to reduce pollen contamination in forest tree seed orchards such as supplemental mass pollination (Korecký and El-Kassaby 2016; Stoehr et al. 2006), water cooling to induce bloom delay (El-Kassaby and Davidson 1991; Song et al. 2018) and greenhouse-like structures (Funda et al. 2016; Moriguchi et al. 2010; Torimaru, Wennström, et al. 2013).

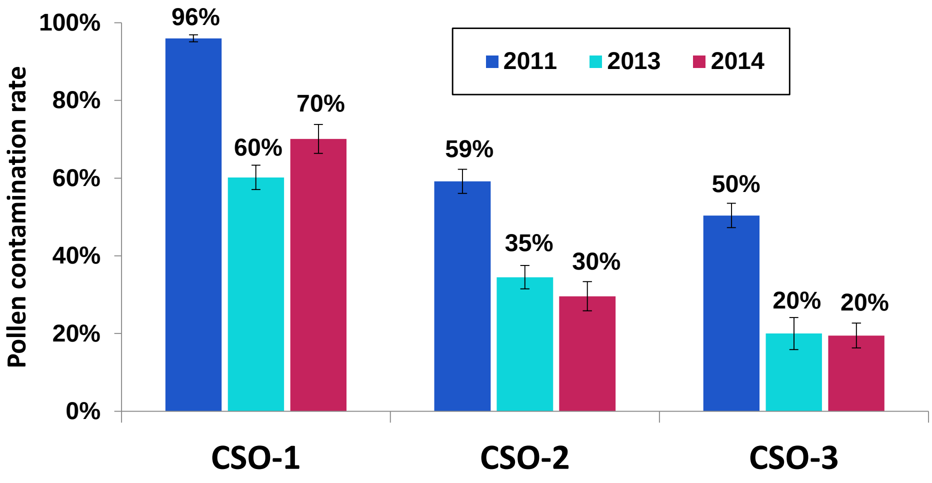

Figure 3. ***Pollen contamination in three VF3 clonal seed orchards*** (CSO-1, CSO-2 and CSO-3) ***per pollination year*** (2011, 2012 and 2013).

In the context of maritime pine seed production, a greenhouse seed orchard would allow for a more intensive management of seed production, with a good control of environmental conditions. For the first time in the maritime pine breeding program, we have tested, in the framework of B4EST project, mass pollination with no flower bagging under greenhouse conditions, to evaluate the technical feasibility of this method, the potential of seed yield per cone and the pollen contamination rate.

3 Maritime pine seed orchard greenhouse experiments

An indoor seed orchard would allow for a more intensive management of seed production, with a good control of environmental conditions. This strategy has been tested before on different species: hybrid larch (Colas et al. 2008), Scots pine (Funda et al. 2016; Torimaru, Wennstrom, et al. 2013), Norway spruce (Johnsen et al. 1994), *Cryptomeria japonica* (Moriguchi et al. 2010), but never on maritime pine. Our objective in task 4.1 (Secure procurement and characterization of improved FRM) is to test on a small scale a greenhouse maritime pine seed orchard, to evaluate the performance of this strategy in improving the quality and quantity of seed production: technical feasibility of this method, potential of seed yield per cone and pollen contamination rate through paternity analysis with an available set of SNP markers.

3.1 Preliminary experiment (pollination done in 2019)

A first set of 20 maritime pine elite genotypes were grafted in 2016 in 100-liter pots and placed in a greenhouse in March 2019 for mass pollination. Due to their young age (3 years after grafting), each tree had only from 0 to 3 female flowers (26 female flowers were counted on the 20 grafted trees in spring 2019). A polymix of two pollens (10-62-1 and 20-67-2) was sprayed three times over the 20 grafted trees in April 2019. After pollination, female flowers were protected against seed bugs with bags and the pots were transferred outside the greenhouse for cone maturation in May 2019. Out of 20 grafted trees, 15 trees had 1 or 2 cones which were collected in December 2020 (19 cones collected in total). Seeds were extracted in January 2021. After elimination of empty seeds (from 21 to 70% of empty seeds per mother tree, 40% in average), a total of 360 seeds remained and were sowed in the nursery in June 2021. Germination rate was highly variable depending on the mother tree (from 0 to 90%, mean germination rate of 70%) and finally 253 seedlings were obtained from 14 grafted trees. Seed production and germination rate for the 20 grafted genotypes are detailed in Table 1.

⇨ A limited number of cones (19 cones) were collected as this preliminary experiment was carried out on young trees (3 years after grafting). However, an **unexpected high level of empty seeds** (40%) associated with a medium level of germination rate (70%) were observed. In total, 605 seeds were collected but only 253 seedlings were viable showing that physical protection (flowers were bagged just after pollination) was not sufficient to preserve high viable seed yield. Other reasons than insect damages could explain low seed yield such as insufficient pollen used for mass-pollination, abiotic stress during cone maturation…

Genotype_name	Female_flower_Nber	Cone_Nber	Seed_Weight (g)	Seed_nber	Empty_Seed_nber	%_Empty_Seed	Sowed_Seed_Nber	Germination rate
9-86-3	1	1	0,12	8	5	63%	**3**	0 (0%)
9-98-4	1	1	1,27	34	16	47%	**18**	15 (83%)
9-117-2	1	1	0,59	17	9	53%	**8**	6 (75%)
16-210-1	1	1	0,68	40	28	70%	**12**	10 (83%)
CORT17	2	2	2,55	48	10	21%	**38**	2 (5%)
507-26-3	1	1	1,10	51	31	61%	**20**	16 (94%)
507-8-1	2	2	1,12	44	26	59%	**18**	15 (83%)
507-26-2	1	1	0,49	21	9	43%	**12**	9 (75%)
507-48-1	2	2	2,60	34	7	21%	**27**	21 (77%)
507-48-2	1	1	1,32	26	9	35%	**17**	10 (59%)
507-49-1	2	2	6,15	122	28	23%	**94**	85 (90%)
507-49-3	1	1	1,07	26	9	35%	**17**	15 (75%)
508-28-2	1	1	0,55	30	14	47%	**16**	9 (56%)
508-53-4	1	1	2,28	84	39	46%	**45**	28 (62%)
509-72-2	1	1	0,59	20	5	25%	**15**	12 (80%)
21-36-4	3	0	-	-	-	-	-	-
508-39-1	1	0	-	-	-	-	-	-
507-52-3	0	0	-	-	-	-	-	-
508-42-4	1	0	-	-	-	-	-	-
508-53-4	2	0	-	-	-	-	-	-
Total	**26**	**19**	**22,48**	**605**	**245**	**40%**	**360**	**253 (70%)**

Table 1. *Number of female flowers, number of cones, number of seeds and germination rate per genotype* from 2019 mass pollination in the greenhouse seed orchard***.***

Genotyping was then performed in order to estimate paternal contribution (two pollens were used for mass pollination) and pollen contamination (even if pollination was performed in a greenhouse, there is a large pollen cloud brought by surrounding maritime pine stands).

Needles were collected for DNA extraction on the 253 seedlings, on the 15 grafted trees and on the two genotypes used in the polymix. Genotyping with 62 polymorphic SNPs was carried out with the Mass Array Technology following Vidal et al. (2015). After filtering, 60 SNPs were retained for the paternal analysis done with the Cervus software (the mean number of SNP available per seedling was 58.8 showing highly successful genotyping). Paternal analysis results are reported in Table 2.

Genotype_name	Number of seedlings	Father 20-67-2	Father 10-62-1	Unknown father (contamination)
9-98-4	15	12 (80%)	2 (13%)	1 (7%)
9-117-2	6	5 (83%)	1 (17%)	0 (0%)
16-210-1	10	8 (80%)	2 (20%)	0 (0%)
CORT17	2	0 (0%)	2 (100%)	0 (0%)
507-26-3	15	12 (80%)	3 (20%)	0 (0%)
507-8-1	15	12 (80%)	3 (20%)	0 (0%)
507-26-2	9	7 (78%)	2 (22%)	0 (0%)
507-48-1	21	14 (67%)	6 (29%)	1 (5%)
507-48-2	10	7 (70%)	3 (30%)	0 (0%)
507-49-1	85	64 (75%)	21 (25%)	0 (0%)
507-49-3	16	11 (69%)	5 (31%)	0 (0%)
508-28-2	9	5 (56%)	4 (44%)	0 (0%)
508-53-4	28	16 (57%)	12 (43%)	0 (0%)
509-72-2	12	9 (75%)	3 (25%)	0 (0%)
Total	**253**	**182 (72%)**	**69 (27%)**	**2 (1%)**

Table 2. ***Paternal contribution and pollen contamination per genotype from 2019 seed production in the greenhouse seed orchard.***

⇨ Very low level of pollen contamination was observed (only 1% of the seedlings were from an unknown father) which demonstrates the high efficiency of mass-pollination in a greenhouse to limit contamination. However, even if the same amount of each pollen was used in the polymix, the pollen 20-67-2 contributes significantly more in the seedlings (the genotype 20-67-2 was identified as the father for 72% of the seedlings vs. 27% for the genotype 10-62-1). This differential contribution is similar whatever the mother considered which suggests possible differences in pollen germination rate or in pollen size.

3.2 Main-mass pollination experiment in 2021

No pollination was done in 2020 due to covid restriction. In 2021, grafted trees from 9 elite genotypes were used to design the main experiment. From 1 to 9 ramets per genotype were available and dispatched into two contrasted treatments: mass-pollination in the greenhouse (treatment M1) and mass-pollination outside the greenhouse (treatment M2), plus a control (M0) which corresponds to grafted trees located outside with no artificial pollination. Table 3 summarizes the ramets distribution in each treatment. A polymix of 3 pollens (8-147-3, 9-42-1 and 9-84-5) was sprayed six times (on 4th, 6th, 8th, 10th, 13th and 15th April 2021) on the grafted trees located either in the greenhouse (M1) or outside (M2). No artificial pollination was applied for ramets used as control (M0) but they were still subject to pollination from surrounding stands. Number of female flowers counted before pollination and number of immature cones observed in autumn 2021 are reported in Table 3. These observations suggest a low cone yield (only 19 cones were counted contrasting with the 82 female flowers observed initially) which could be due to late frost that occurred in spring 2021.

Genotype_name	Ramet_Nber				Female_Flower_Nber				Cone_Nber			
	M0	M1	M2	Total	M0	M1	M2	Total	M0	M1	M2	Total
3814-2	1	4	4	9	1	14	13	28	0	1	0	1
F1.1678	1	4	4	9	3	11	10	24	3	1	3	7
10-54-2	1	2	2	5	1	5	3	9	0	2	0	2
10-72-2	0	2	2	4	0	4	4	8	0	1	1	2
F1.0756	0	1	1	2	0	3	1	4	0	1	0	1
10-138-1	0	1	1	2	0	2	1	3	0	2	1	3
10-52-2	1	1	0	2	1	2	0	3	0	2	0	2
19-70-3	0	1	1	2	0	1	1	2	0	1	0	1
10-63-2	1	0	0	1	1	0	0	1	0	0	0	0
⇨ **Total**	**5**	**16**	**15**	**36**	**7**	**42**	**33**	**82**	**3**	**11**	**5**	**19**

Table 3. *Repartition of ramets per treatment (M0, M1, M2), number of female flowers and cone yield for each genotype* in the mass pollination experiment done in 2021.

Mature cones will be collected in December 2022 after the end of B4EST project. Seeds will be extracted and sorted by ventilation in order to estimate seed yield and rate of empty seeds. This seedlot will be genotyped in a future project for paternity analyses. The objective will be to estimate efficiency (viable seed yield, rate of pollen contamination, paternal contributions) of mass-pollination in greenhouse (M1) and outside (M2) in comparison to open-pollination (M0).

4 Perspectives and recommendations

The set of molecular markers developed by Vidal et al. (2015) for maritime pine are now routinely available for seed orchard analyses. Such markers were used to study pollen contamination in three maritime pine seed orchards. Results highlight the importance of seed orchard location and seed orchard age to avoid pollen contamination. This study could be extended to analyze seedlots collected over successive years in order to better understand the influence of annual climatic conditions on pollen contamination and seed yield.

In the present study, the same markers set was considered to evaluate efficiency of mass-pollination. The pollination for the main experiment was delayed by one year due to covid restriction. In consequence, only seeds from the preliminary experiment were available. The seedlots from the main experiment will be available after the end of B4EST project in 2023.

The preliminary experiment confirms the low seed yield observed in seed orchards, even though pollinated flowers and conelets were protected with bags suggesting multifactorial causes of cone abortion and empty seeds. Researches related to the drop in seed production due to biotic and abiotic stresses must be strengthened in order to make recommendations for seed orchard managers. The preliminary experiment demonstrates also the efficiency of mass-pollination under a greenhouse to avoid pollen contamination as already observed by Funda et al. (2016) and Moriguchi et al. (2010). Paternal contribution could be more balanced if pollen germination rate is controlled when designing the polymix. No self-fertilisation was observed in this experiment, as young grafted trees did not produce pollen. The future analysis of the seedlots from the main experiment will allow to estimate the efficiency of mass-pollination when realized outside the greenhouse.

The young age of grafted trees used for this experiment limits the seed production as only few female flowers per tree were available for pollination. Seed production should increase in the coming years but pruning will be necessary to be able to handle trees in the greenhouse.

Pruning skills for maritime pine does not currently exist in our institute, which means that technical development would be necessary if greenhouse seed orchards are to be deployed more extensively. Further needs will also concern evaluation of cost of establishment and management of greenhouse seed orchard for maritime pine as this experiment was realized at a very limited scale with young grafted trees.

5 Dissemination activities

First results provided by this greenhouse experiment have been shared in the maritime pine breeding community in France (GIS Pin maritime du future). This experimentation will continue thanks to financial supports for regional and national programmes considering the importance of seed production in the context of increased needs at both regional (impact of 2022 fire damages) and national levels (extension of maritime pine area in northern areas in France).

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