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Master thesis

Impact of the environment on the quality of the seeds of Douglas-fir seed orchards

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PRESENTATION OF THE COMPANY

National Forests Office (ONF) is a public establishment of the National Government of France charged with the management of national forests. It was established in 1966 and its headquarter is situated in Paris. Its nature is public, industrial and commercial, conducting its work in a framework of a multi-year contract of objectives and acting with the State. The main task is management of French public forests which cover the surface of nearly 10 million hectares of forests and woodland in Metropolitan France (1/3 French forests) and overseas departments. The main missions and activities are:

- ONF ensures 40% of timber market in France, ensuring renewal of public forests and proper maintenance of their stands;
- biodiversity preservation and increase, through its inclusion in current forest management, but also because of the importance of Natura 2000 network in public forests and the extension of its biological reserves network;
- offers new types of forests, by adapting to the diverse public expectations and different contexts (suburban forests, tourist areas), while raising awareness servicing missions: forest renewal and biodiversity preservation;
- performs services for communities and private clients based on a decentralized territorial organization, work agencies, consulting firms and specialized skills networks
- provides public service missions for the prevention and management of natural risks and the preservation of biodiversity;
- boosts the role of the forest and the "wood products" in the fight against climate change.

With the aim of ensuring sustainable forest management towards better quality and protection of the natural environment, the ONF's environmental policy is built around five themes:

- **biodiversity** as it is an essential factor of stability, resilience and ecosystem restoration, and because its preservation in terms of habitats, species and genetic resources is a global issue;
- **water**, for its role in public health and because it is an indispensable resource, and supports rich and strategic environments for the proper functioning of ecological cycles;
- **ground** because it is the basis of forest sustainability and productivity and because protection against erosion is a major issue for prevention of natural risks;

- **landscape**, because the forest participates in our living environment, contributing to its variety and is a place of healing and privileged activities of urban and rural areas;
- **eco-responsibility**, because the ONF must actively contribute to conserving natural resources, participate in the fight against the greenhouse effect and improve the environment, with the involvement of all staff, through its policy of sustainable purchasing and commitment to achieve energy savings and reduce consumables and waste.

In 1997, ONF has begun with its international activities to enhance its expertise creating subsidiary ONFI (ONF International). It is present in Latin America (Brazil, Chile, Columbia) and Africa (Gabon, Cameroon). In Brazil, it was founded in 1999, in partnership with Peugeot and Pro Natura, with the main goal to protect Amazonian rainforests.

INTRODUCTION

Seed orchards constitute the link between tree breeding and plantation forestry (Webber, 1994). Although they are not the only way of transferring genetically improved traits to forests, they are the most widely used way. The frequency of desirable genes in the population is increased progressively through cycles of selection and crossing. That process involves sexual reproduction for progeny testing. Since the most forest tree species are established with seedlings, seeds have been established as a major output from genetic improvement programs (Sweet, 1994). Gene flow in the seed orchards provides an insight into prevailing population dynamics and it is one of the main factors determining the genetic architecture of populations. (Burczyk, 2004). Gene flow patterns and seed quality appear to be the main focuses of orchard improvement.

This Master Thesis was part of the QuaSeGraine project which is collaboration between Office National des Forets (ONF) and Institut National de la Recherche Agronomique (INRA). The brief description would be: quality estimation and selection of seeds of economically interesting softwood tree species (Douglas-fir, larch and maritime pine) depending on the orchard structure and environmental factors. The main objectives of this study were to determine whether changes caused by environmental factors have a real commercially applicable effect on the quality of the Douglas-fir seed and to determine to what extent the gene flow patterns and orchard characteristics affect the genetic superiority of the seed orchard.

The ONF has a research and development and Innovation department whose one of the goals is to insure forest adaptation to climate changes. One of the levers as possible is the right choice of plant material.

PART I

1. BIBLIOGRAPHIC STUDY

1.1. Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco)

Pseudotsuga menziesii (Mirb.) Franco, commonly known as Douglas-fir, is an ecologically and economically important timber species. With the natural distribution range in North America and Canada, it became of enormous importance in European forestry in the second half of twentieth century. There are two varieties in the natural range: coastal variety (along the Pacific coast) *P. menziesii*, var. *menziesii,* and interior variety *P. menziesii*, var. *glauca*. First specimen was planted in Scotland as an ornamental tree, and after World War II, it became major reforestation species. It is popular mostly due to its use as a high-quality timber. From a forest point of view, Douglas-fir is important as a reforestation species because of its fast growth and strong resistance to diseases and insects (Paques, 2013).

The largest Douglas-fir seed producing plantations in Europe are in Germany (3,030 ha), France (421 ha), Italy (259 ha) and Belgium (256.3 ha). Ever since its plantations covered great surfaces in Europe, there was an expressed need for deeper understanding on ecology, genetics and forestry for this introduced species. Since the European environment differs from Douglas-fir natural stands, it was important to determine how to choose the right reproductive material for reforestation. Forest managers need highest seed quality for their pedo-climatic environment. Two possibilities are used for stand renewal: natural regeneration or plantation. In both cases, the best equilibrium between growth and wood quality is researched. The most of the old Douglas-fir stands in Europe are the result of the material of unknown origin. For most of them, no information from the geographical harvest stand, number of trees collected or genetic diversity level are available. So, the quality of the seedlings is uncertain and the natural regeneration quality could be worst for the adult stand (mortality, form default, early flushing…). To mitigate this uncertainty, breeders have selected the best phenotypes in several forests to create seed orchards which produce seeds better adapted to French conditions. Such breeding populations are managed to maintain higher levels of genetic variability for sustained long-term genetic response (Namkoog, 1988).

Moreover, climate change is expected to cause substantial changes in tree growth by altering growing season length, soil moisture availability, and other climate-related factors

important to plant life cycle (Davis and Shaw, 2001). To understand how the forest trees could be adapted to such changes would be very useful for forest managers.

1.2. Seed orchards

Tree breeding and plantation forestry are linked with the concept of producing genetically superior conifer seed which is established as the main objective of tree improvement programs. These programs are expected to provide genetically superior seed for reforestation programs and future harvest (Kess, 2015). The main objective of seed orchard can be met if the criteria for panmictic equilibrium are fulfilled: 1) completely random fertilization; 2) equal number of male gametes/clone for all clones; 3) equal number of female gametes for all clones; 4) lack of genetic barriers that may affect embryo viability; 5) lack of pollen contamination; 6) equal rate of self-fertility for all clones in the seed orchard (Alizoti, 2009).

As the environment of the most of the seed orchards cannot be fully controlled, not all of the aforementioned criteria can be met. However, substantial research has been dedicated to illustrate that seed orchards have never met panmictic population (El-Kassaby and Askew, 1998). Several factors often compromise estimates of seed orchards being perfect, closed populations. These factors are: fertility variation, reproductive phenology asynchrony and minimal isolation from undesirable gene flow (pollen contamination).

1.3. Gene flow and pollen contamination

The most tree breeding programs tend to increase the economic value of specific traits of interest while maintaining conservation of potentially advantageous genes. Gene flow, therefore, appears to be the main problem contrasted to the tendencies of the most tree breeding programs, It is the only external factor affecting seed crops' genetic quality with estimates ranging from nearly 0% (El-Kassaby, 1986) to 90% (Fast, 1986). Gene flow determines the genetic architecture of populations, along with mutation, drift and selection (Burczyk, 2004).

Gene flow has prominent roles in evolutionary biology and ecology. In conservation biology, it has a big influence on the effective population size of threatened species (Lande, 1988). From ecological point of view, it is one of the key factors determining composition of ecosystems and responses to disturbance (Conell and Slatyer, 1977). In addition to this, gene flow has gained substantial interest in applied fields like crop improvement and ecological risk assessment (Burczyk, 2004). The effects of pollen contamination on seed crops can be

deleterious (Bateman, 1947). Along with reduced mating success and pollen contamination from outside sources, these deleterious effects have an impact of realized gains in seed quality (DiGiovani and Kevan, 1991). Generally, it is assumed that pollen from outside orchards has a negative effect on progeny performance as reduced growth potential and (or) maladaptation (Stoehr, 2004). However, external source pollen can sometimes have a positive effect. For example, if a high elevation seed orchard is situated at a lower elevation, then the seedlings resulting from contaminant pollen can show faster growth (Stoehr, 2004).

Gene flow in terms of pollen contamination can vary significantly depending on the age of a seed orchard and on the spatial distance from external pollen sources. In a study conducted in 1983 by Smith and Adams, the authors concluded that the high level of contamination was not, indeed, surprising, because the orchard was of very young age (14 years from grafting). Furthermore, it was not isolated from natural stands of Douglas-fir. They predicted that as the orchard blocks matured and pollen production within blocks increased, pollen contamination would diminish. Methods of measuring and managing pollen contamination are very important to tree breeders (Webber and Painter, 1996). Self-fertilization and unequal contributions of parents to seed crops also reduce the genetic efficiency of seed orchards (Friedmann and Adams, 1985).

In order to determine the influence of pollen contamination on genetical variability of a seed orchard and its impact on the seed quality, indirect methods can be used. Simple sequence repeats have already been used to measure gene flow, pollen contamination and selfing rates, directly quantifying the proportion of seeds fathered by each seed orchard parent and testing for deviations from random mating with respect to distance between orchard trees (Stoehr and Newton, 2002; Slavov, 2004). The information provided by these studies can further be utilized in forest management practices to advise them about the tree numbers which must be harvested for the guarantee of high genetic diversity.

1.4. Reproductive phenology

Reproductive phenology is considered to be one of the main factors causing panmictic disequilibrium. Along with cone production levels, level of contamination from outside sources, orchard planting arrangements, the use of supplemental mass pollination (SMP), and water spray cooling treatment to delay reproductive bud opening, it represents biological factors and

management practices which affect the mating of seed orchard trees. The quality of produced seed depends on the mating system in the seed orchard (El-Kassaby, 1986). Another possibility is to select trees with the same phenology which allows them to interpollinate.

Regarding phenological stages, trees can be divided into three classes: early, intermediate and late phenological class. El-Kassaby and Ritland in their 1986's study showed the importance which different phenological stages have on the final contamination and self-fertilization rates. It was concluded that the highest pollen contamination occurs in early phenological class. It is mostly due to the fact that the pollen of inside origin is outnumbered in concentration by the pollen coming from outside sources during early flushing. When SMP practice is applied, the level of outcrossing decreases and it reduces the extent of pollen contamination.

Variation in reproductive bud development and overlap between reproductive bud flush of individual trees can decrease seed yield affecting both the quality and quantity of the seeds and consequentially reduce the effective population size. Since tree phenology has an impact on seed quality, seed yields and the size of effective orchard population (El-Kassaby, 1983), it is important to determine how these phenological properties affect genetic variability of seed orchard population.

1.5. Genetic analysis in tree breeding

In order to determine pollen contamination rate and, accordingly, the seed quality depending on genetic diversity, several molecular methods have been used so far. At the very beginning, molecular markers such as isozymes or allozymes were utilized (Squillace and Long 1981; Friedmann and Adams 1985). Because isozymes show low effective numbers of alleles per locus, it is not possible to detect all the male contributors or to characterize unambiguously mating gene flow within orchards (Adams, 1992).

Highly variable, polymerase chain reaction (PCR) based genetic markers, such as simple sequence repeats (SSRs), can substantially increase the precision of pollen contamination estimates by increasing detection probabilities (Dow and Ashley 1998; Gerber, 2000).

Simple sequence repeats (SSRs or microsatellites) are a class of molecular markers based on tandem repeats of short (2-6) DNA sequences (Litt and Lutty, 1989). The most abundant in Douglas-fir and several others conifer tree species are found to be AG, AC and ATG among di-, tri- and tetranucleotide SSRs (Amarasinghe, 2002). The high variability, reproducibilty, and codominant nature of SSR markers makes them very valuable tools for genotype identification,

parentage analysis, and genome mapping in Douglas-fir and other conifer tree species, especially because of their large and complex genomes (Slavov, 2004). These markers have already been used to measure pollen contamination and selfing rates, directly quantifying the proportion of seeds fathered by each seed orchard parent, and testing for deviations from random mating with respect to distance between orchard trees (Stoehr and Newton 2002; Chaix, 2003; Slavov, 2004).

1.6. The objectives of this study

Studying contemporary gene flow provides insights in current population genetics (Sork, 1999). Pollen dispersal patterns, gene flow between populations and differential reproductive success can influence the levels of inbreeding, effective population size and distribution of genetic diversity between and within populations (Burczyk, 2004). Although pollen contamination, resulting in gene flow, is generally considered undesirable, it can have some positive effects on seed orchards, as previously stated (Stoehr, 2004). However, pollen contamination along with variations in reproductive bud development and differential pollen shedding periods can affect the seed orchard seed quality and quantity by reducing the breeding population size and by lowering seed yield (El-Kassaby, 1983). It is, therefore necessary to determine the extent of pollen contamination in the seed orchard and to achieve synchrony in bud flushing and pollen shedding in order to achieve the high-quality and genetically improved seed.

The main objectives of the conducted study were:

1. Compare genetic diversity of progenies and parental population
2. Determine the relative paternal contribution of clones in seed orchard;
3. Determine the effect of the microenvironment of each clone on the paternal contribution rates;
4. Estimate the effect of potential fathers phenology on assortative mating.

2. RESULTS

2.1. Genetic characterization of adult and progeny population

For the genetic analysis of two populations, adult and progenies, 11 microsatellite markers were used.

❖ Adult population

For the population of adults, which is represented by one ramet by clone, the number of alleles (N_a) varied between 17 (PmOSU_4G2) and 41 (PmOSU_1F9), with an average value of 27.818 and standard error (SE) value of 2.381. The number of efficient alleles (N_e) varied between 6.162 (PmOSU_3G9) and 20.175 (PmOSU_1F9), with an average of 13.329 and SE value of 1.294.

Genetic diversity can be estimated through the parameter of expected heterozygosity which varied between 0.838 (PmOSU_3G9) and 0.950 (PmOSU_1F9), mean value of 0.916 and SE of 0.01. The value of expected heterozygosity is higher than the value of observed heterozygosity, for example for marker PmOSU_3F1 whose *F*-index value is 0,254 and it represents significant heterozygosity deficit. The statistically significant heterozygosity deficit was observed for all the markers, except for PmOSU_2G12, PmOSU_4A7 and PmOSU_2C2.

❖ Progeny population

For this analysis, we used all 43 progenies. A higher number of different alleles was counted for progeny population, the lowest being 20 and the highest 44 with the mean value of 29.273 and SE value of 2.367 than for adult population. The number of efficient alleles was between 5,483 (PmOSU_3G9) and 13,434 (PmOSU_4A7), average value was 10,528 and SE 0,819. The expected heterozygosity was in the range between 0,818 (PmOSU_3G9) and 0,926 (PmOSU_1F9) with the average value of 0,897and SE of 0,010. *F*-index was between -0,012 (PmOSU_1F9) and 0,514 (PmOSU_3B2) with mean value of 0,212and SE of 0,058.

We also analyzed separately each progeny, changes could be observed in observed and expected heterozygosity. In that case, some of the populations do not show heterozygosity deficit.

❖ Comparison between adult and progeny population

The genetic diversity of the progeny, as measured by observed heterozygosity and number of alleles per locus differed from adult population (Table 1). Mean H_o in adult and progeny is 0,708 ± 0,054 and 0,699 ± 0,056, respectively, indicating a slight reduction in genetic diversity. While mean diversity decreased, observed heterozygosity at several loci (PmOSU_2G12, PmOSU_3B2, PmOSU_2C3, PmOSU_4A7) increased in progeny population.

Expected heterozygosity at all loci is higher than observed heterozygosity, indicating deviation from random mating in both populations. Gain of alleles was observed at all loci expect PmOSU_2G12 and PmOSU_4A7 which indicated genes from outside of the seed orchard.

Table 1. Genetic diversity of adult and progeny populations, indicated by number of alleles, observed heterozygosity (H_o) and expected heterozygosity (H_e).

	Adults				**Progenies**			
Locus	***N*a**	***H*o**	***H*e**	***F***	***N*a**	***H*o**	***H*e**	***F***
PmOSU_2G12	20,000	0,847	0,910	0,017	19,000	0,911	0,927	0,069
PmOSU_3B2	32,000	0,448	0,922	0,493	33,000	0,477	0,940	0,514
PmOSU_3D5	22,000	0,589	0,893	0,384	28,000	0,561	0,910	0,340
PmOSU_2C3	32,000	0,499	0,924	0,398	33,000	0,569	0,946	0,460
PmOSU_3F1	28,000	0,732	0,915	0,254	30,000	0,695	0,932	0,200
PmOSU_3G9	20,000	0,485	0,818	0,546	21,000	0,381	0,838	0,406
PmOSU_4A7	36,000	0,847	0,925	0,073	31,000	0,866	0,934	0,084
PmOSU_1F9	41,000	0,937	0,926	0,050	44,000	0,903	0,950	-0,012
PmOSU_2C2	23,000	0,867	0,892	0,062	26,000	0,837	0,892	0,028
PmOSU_2D4	35,000	0,677	0,891	0,310	37,000	0,636	0,922	0,240
PmOSU_4G2	17,000	0,859	0,855	0,034	20,000	0,852	0,882	-0,005
Mean	27,818	0,708	0,897	0,238	29,273	0,699	0,916	0,212
SE	2,381	0,054	0,010	0,060	2,297	0,056	0,010	0,058

The consanguinity coefficient is always positive in all loci for adult population while this is not the case for two loci in progeny population. The value of F_{ST} was 0.004 and ± 0.000 which indicated no difference between the populations.

The frequencies of observed null alleles per each locus are shown in Table 2.

Table 2. Null allele frequencies for progeny population. Ns-not significant; nd-non-defined; **p<0.01; ***p<0.001.

Locus	Frequency	P value
PmOSU_2G12	0,0142	Ns
PmOSU_3B2	0,3659	Nd
PmOSU_3D5	0,2804	Nd
PmOSU_2C3	0,3093	Nd
PmOSU_3F1	0,0806	***
PmOSU_3G9	0,2529	Nd
PmOSU_4A7	0,028	***
PmOSU_1F9	0,0301	***
PmOSU_2C2	0,0152	Ns
PmOSU_2D4	0,1103	***
PmOSU_4G2	0,0043	**

Null alleles were observed at all loci. The highest and lowest frequency was observed in PmOSU_3B2 and PmOSU_4G2 respectively. Except for PmOSU_2G12 and PmOSU_2C2, the P value was significant for all of them. These high null allele frequencies could have interfered with parentage analysis.

2.2. Paternal assignment

Allele frequency analysis and simulations of parentage assignment were conducted with Cervus 3.03 at the 11 loci used for this study. These loci show sufficient diversity for parentage analysis based on high average polymorphic information content (Botstein, 1980), low cumulative probability of incorrect parentage assignment, both interfered with allele frequency analysis and parentage assignment success in simulations of parentage assignment in Cervus 3.03.

Polymorphic information content (PIC) was greater than 0.80 per loci with a mean PIC value of 0.8924 across loci. Non-exclusion probability for all parents was very low, the probability of erroneous parentage assignment for the 11 loci was $1.395\ 10^{-14}$ which indicated that genotyping of individuals with less loci allowed in this study was still sufficient for correct parentage assignment.

Table 3: PIC and parent pair non exclusion probability (NE-PP) of microsatellite loci used for parentage analysis

Locus	PIC	NE-PP
PmOSU_2G12	0.906	0.051
PmOSU_3B2	0.920	0.037
PmOSU_3D5	0.888	0.064
PmOSU_2C3	0.923	0.035
PmOSU_3F1	0.912	0.044
PmOSU_3G9	0.803	0.149
PmOSU_4A7	0.922	0.035
PmOSU_1F9	0.925	0.033
PmOSU_2C2	0.884	0.071
PmOSU_2D4	0.888	0.058
PmOSU_4G2	0.845	0.109
Mean	0.8924	NA

Accurate measure of parental reproductive success is a prerequisite for understanding true pollination dynamics in this seed orchard. Using CERVUS software, the pedigree reconstruction successfully assigned the paternal parent to the analyzed seedlings: 95 fathers out of possible 134 were assigned to 43 progenies. Pollen was contributed by 70% of clones, highlighting paternal reproductive success. However, we found 4 seeds with no seed orchard's paternal donor which could be correlated with gain of alleles in progenies.

The number of different fathers in ramets of different clones varied between 5 and 24. Eleven progenies collected more than 20 different pollen donors whereas only four progenies were pollinated by less than 10 pollen donors. Furthermore, one pollen donor was present in 13 analyzed progenies and the least abundant fathers were present in only one clone. 27.9% of

clones showed selfing which represents 3% of all seeds. It is the early flushing clone 272 which has the largest rate of inbreeding.

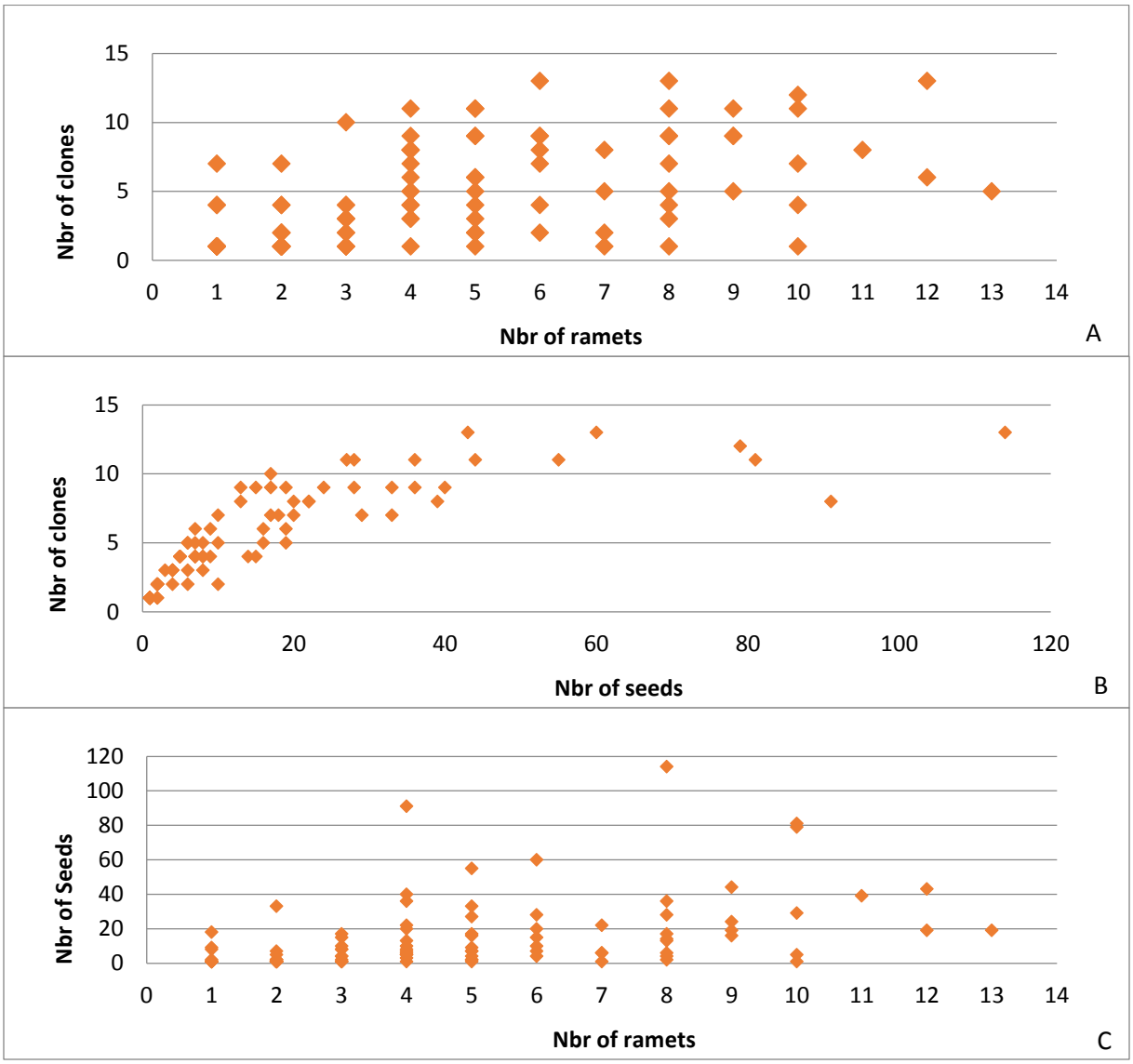


Figure 1. The figure shows how the parameters describing fathers in the seed orchard affect the genetic diversity of the progeny. A illustrates the effect of the paternal contribution on the genetic diversity of the progeny. B and C show that the fathers represented with higher number of ramets can fertilize more seeds originating from more different clones. For fathers represented by seven or nine ramets, the values of fertilized seeds and clones are below the observed general trend.

In assignment analysis, we determined its paternal contribution in each progeny, its abundance in the seed orchards (number of copy of each clone) and its overall seed abundance in the orchard. Figure 4 shows how these parameters affect gene flows in the seed orchard.

If the pollen donor has numerous copies in the seed orchard, it can pollinate more different clones (Fig 1A). These indices could be a good proxy for the diversity of pollen cloud

for tree breeders. The figure 1B shows that pollen donors which are good seed contributors can distribute their pollen more efficiently on different clones. Moreover, the higher number of copies of a certain clone means that this clone will be greater paternal contributor of the seeds (Fig 1C). The more abundant fathers will contribute more than less abundant individuals to the next generation. There are some deviations from the observed trend which might be the consequence of the spatial distribution of the paternal clone or of the phenology.

2.3. The effect of phenology

In order to determine the phenology stage effect on genetic diversity and paternal contribution to the progeny, the phenology stage was assigned to each individual in the seed orchard. The flushing patterns of analyzed trees might explain why some fathers are represented in high number of different clones while some are present in only one clone. We also expected that the most of the clones flushing in same time have higher chance of possible crossings. The clones which were represented by several copies show at least three different phenotypical stages. Only on the second date we have seen the diversity of phenological stages (Fig 2).

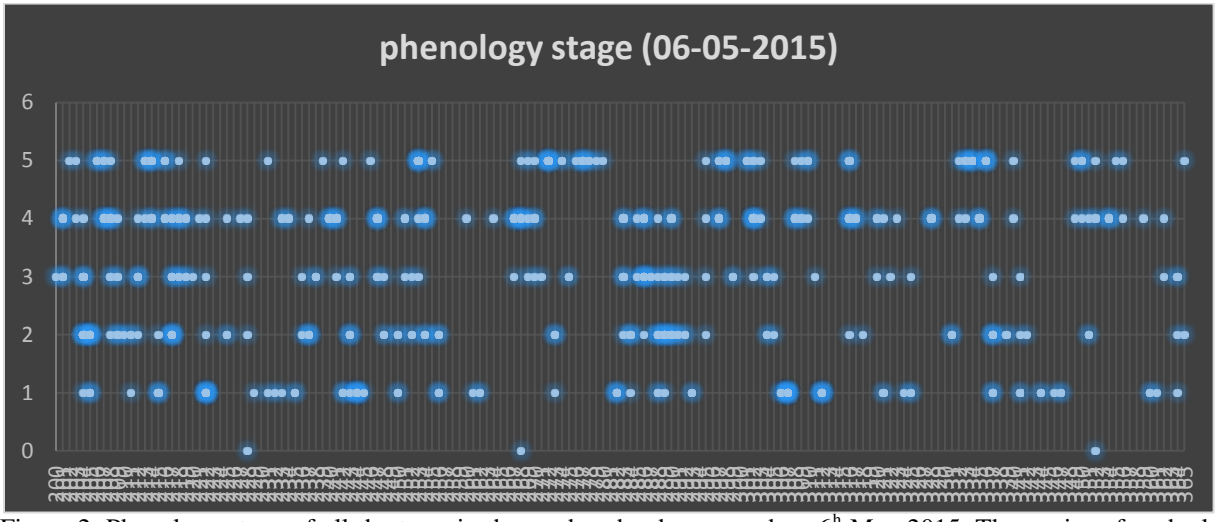


Figure 2. Phenology stage of all the trees in the seed orchard measured on 6^{h} May 2015. The copies of each clone are represented with the blue points designated to certain flushing stage. The brightness and the diameter of the point are the measure of the number of copies with the same flushing state value.

Of all the analyzed fathers, only clone 272 is an early flusher (Fig 3). It has the greatest paternity contribution, clonal diversity of fertilized seeds and abundance in the seed orchard, which is not expected. But it could be explained by a fast flowering period.

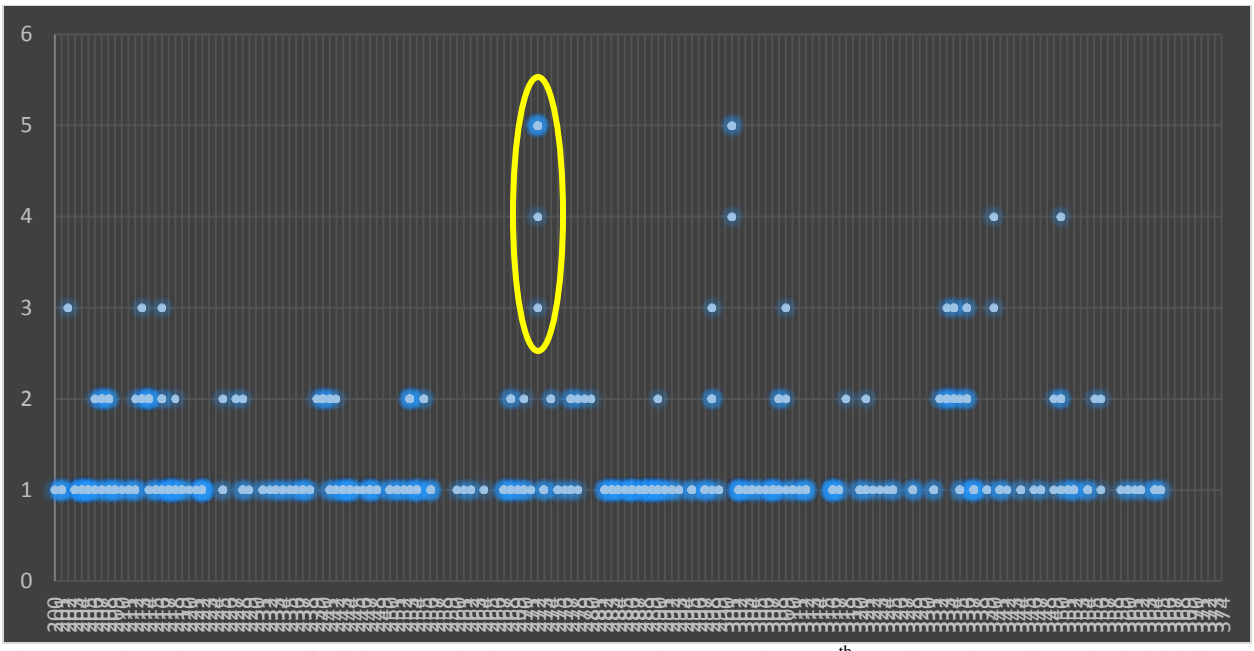


Figure 3. Phenology stage of all the trees in the seed orchard measured on 24th April 2015. Yellow circle highlights the clone 272 with the highest paternal contribution, greatest clonal diversity and the most abundant clone in the seed orchard.

2.4. The impact of the environment

Each clone should be surrounded by 8 other clones but due to mortality it was not always the case. We collected information on the local environment of each seed source clone. With the determined father clones in the orchard and the number of trees surrounding each maternal clone, we tried to determine the effect of the environment on the genetic diversity of progeny. From the results shown in Figure 4 it can be concluded that the progeny diversity increases proportionally to the number of its neighbouring trees. However, several individuals seem to fall out of the observed trend (encircled points in the Figure 4). Different values of their expected heterozygosity might be due to their placement in the seed orchard or phenology asynchrony.

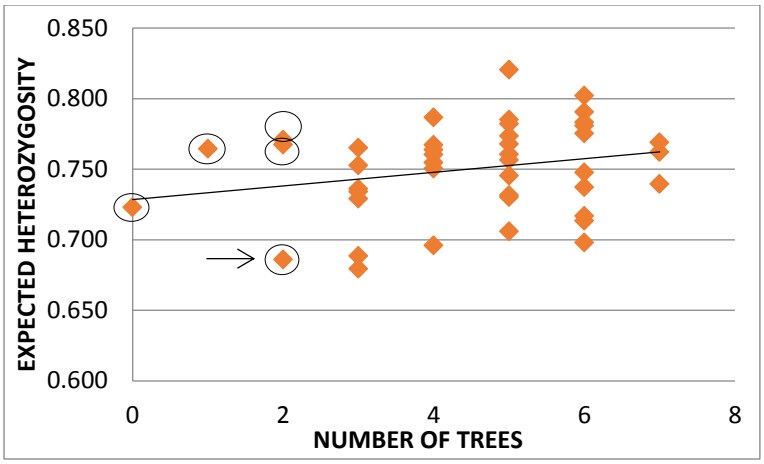


Figure 4. The figure shows the dependence of heterozygosity of an individual on the number of surrounding trees. Each spot represents an analyzed ramet of a particular clone in a seed orchard. The observed trend shows that more diverse environment will result in higher heterozygosity. The encircled spots represent the individuals whose deviations are the consequence of their placement in the seed orchard.

3. DISCUSSION AND CONCLUSION

3.1. The quality of genetic markers

The high variability of SSR markers that we used allowed us the precisely estimate gene flow. Compared to using isozyme markers, less effort is needed to verify genotypes and controlled crosses between selected parents. SSRs allow cheap and precise estimation of pollen and seed contamination in seedlots. They also facilitate the estimation of the success of seed orchard management techniques such as bloom delay and supplemental mass pollination. Finally, the high polymorphism of these markers can be used to directly determine the relative maternal and paternal contributions in open-pollinated seedlots from seed orchards.

However, in this study, the excess of homozygotes was observed for all the analyzed markers in progeny population. In adult population the excess was significant in 5 of the 11 utilized markers. The mean fixation indices F (F_{IS}) were significantly different from Hardy–Weinberg expectation. This difference attests that a high frequency of null alleles and allelic dropout in the SSR loci were the major reasons for false homozygote genotyping that inflated fixation indices in our study (e.g., Pompanon, 2005). Allelic dropout and null alleles are the most prevalent source of genotyping error in general (Miller, 2002; Dufang, 2015). Null alleles, allelic dropout, and stutter bands have all been observed in other studies using microsatellite loci from this marker set (Krutovsky, 2009; Fussi, 2013). The existence of null alleles was previously mentioned by Slavov et *al*. (2004), but only for the PmOSU_3F1 marker and at higher rates. In order to reduce the possible genotyping errors, we obtained several laboratory practices. PCR

controls were used to reduce the errors which might have been caused by reading allele size slippage. Also, we used the same concentrations of DNA to avoid preferential amplification of small alleles. However, possible genotyping errors cannot be completely avoided in this way. Slippage during amplification can cause microsatellite sizes which are different from their original size, with one repeat more or less (stuttering, Shinde, 2003).

Another possible way of explaining the excess of homozygotes is the Wahlund effect. Indeed, large populations could contain sub-populations. Then, there are fewer homozygotes than in the average for the set of subdivided populations. A consequence of the Wahlund effect is that when a number of previously subdivided populations merge together, the frequency of homozygotes decreases. This geographical structure affects allele frequencies over space and consequently the proportions of different genotypes in the local populations. The seed orchard's clones were selected in different populations and could be considered as a set of subdivided population.

Consanguinity is another possible explanation of the high level of homozygotes. Available data from other conifers suggests that homozygote excess, due to selfing or consanguineous mating in seed orchards, present at the embryo stage had been removed after a few years of field growth (Neale, 1985; Yazdani, 1985; Gomory, 1992). Nevertheless, we cannot exclude this possibility because in our seed orchard some clones are originated from the same progenies and could be full-sibs. These clones are suspectible to inbreeding what could produce more related seeds than expected and decrease the level of heterozygosity.

3.2 Changes in diversity between population

Multiple processes cause changes in allele frequencies and observed genetic diversity between generations. In studied seed orchard, high pollen flow and relatively equal parental contributions acted to increase diversity, while high selfing rates and random sampling of alleles between generations acted to diminish some expected gain in diversity.

In our case, negative change in means of heterozygosity was observed in progenies. Gain and loss of alleles were also observed across loci, with a greater number of alleles being gained than lost. Gain of alleles across loci is expected as a result of pollen flow from outside-orchard sources, introducing novel alleles and genotypes. The observed loss of alleles could be due to

self-fertilization, decreased gametic contribution of adults carrying these alleles or sub-sampling of progeny seeds. Our results showed that not all the clones in the seed orchard participate in the orchard gene flow (39 ramets do not participate).

Nevertheless, the comparison of the two populations (adults and progenies) shows F_{st} equal to 0.004, which is really weak.

3.3. Pollen contamination

Only 4 seeds could not be assigned in our study what is very weak and in accordance with the previous study of this seed orchard. But this result is not consistent with pollen contamination levels reported earlier for seed orchards of Douglas-fir and other conifers (Adams and Burczyk, 2000; Pakkanen, 2000). The difference could be due to the substantial spatial isolation from other stands of the same species, and a very good pollen management techniques. Indeed, as shown in the figure 5A most of the clones could have fertilized each other. In our seed orchard there is a pollen saturation of all the clones by these clones, which did not allow fertilization with exogenous pollen.

3.4. Parentage analysis

Low selfing rates are typically detected in seed orchards of Douglas-fir and other conifers (Adams and Birkes, 1991; Stoehr, 1998; Stoehr and Newton, 2002). Our results agree with these findings. Although self-pollination can be as high as 50% in Douglas-fir, severe inbreeding depression during seed development reduces selfing at the developed seed stage to levels that do not appear to be a serious problem for the production of seed lots with high genetic quality (Sorensen, 1999). Thus, self-pollination has a greater negative impact on seed yields than on the genetic efficiency of Douglas-fir seed orchards. The clone which showed highest selfing rate is early flushing.

The abundance of paternal ramets affects the paternal contribution to the genetic diversity of the seed orchard. It plays important role in the clonal diversity of its progeny, but the male flowering has an impact, too. The similar impact of flowering on male mating success was previously detected by Erickson et *al.,* 1998 and Burczyk et *al.,* 1997. The individuals whose flowering is very early or very late have a significantly reduced mating success (Burczyk, 1997). It can be also stated that the father clones that are placed on the borders of the seed orchard or are isolated have a less chance of participating in the progeny.

3.5. Effect of the local tree density on the gene flow

When there are fewer nearby trees available as a pollen donor, the proportion of pollen from distant trees increases in the local pollen pool, and consequently the average realized pollination distance will be larger (Robledo-Arnuncio, 2004; Bacles and Ennos, 2008; Wang, 2010). Based on parentage assignment, Robledo-Arnuncio et al., (2004) demonstrated that the Euclidean distances between pollen donors and mother trees were strongly negatively correlated with the tree density of the forest stands. It has been shown before in *Fraxinus excelsior*, *Q. robur* and *Q. petraea* that low conspecific tree density increased the average distance between mates (Robledo-Arnuncio, 2004; Bacles and Ennos, 2008; Wang, 2010). The most likely explanation for this observation is that pollen competition between distant and nearby trees decreases when there are fewer nearby trees available as a pollen donor, through which the average realized pollination distance will be greater. Furthermore, in more open landscapes, airborne pollen movement will be facilitated, as winds in open landscapes are typically stronger than winds in a canopy closed forest matrix (Nathan, 2002; Bacles, 2005).

The obtained results showed that the individuals which have a crowded surrounding have a greater chance of producing more genetically diverse progeny. If an individual tree in a seed orchard is surrounded by many other trees, there is a higher chance that the fertilizing pollen will originate from its closest neighbours. On the other hand, the pollen cloud which is produced by tree´s closest neighbours represents certain shield which prevents pollen from more distant trees to fertilize them. But, the trees that are in close proximity to them represent sufficiently high pool of genetic diversity so that their progeny can keep high levels of heterozygosity. Although tree isolation in orchard is reflected in their low genetic diversity and higher selfing-rate, our results showed that certain rate of spatial isolation (Annex 1) enables fertilization with more diverse pollen from all over the orchard. Because there is no pollen shield around, pollen from more distant ramets can reach those isolated individuals. However, if isolated individuals have low level of heterozygosity in their progeny, the main cause might be the flowering asynchrony with the most of the orchard.

Conclusion

Production of improved seed for reforestation relies on seed orchards functioning as closed, perfect populations, so genetic gain and diversity are packaged and delivered in terms of

improved seed and seedlings. Deviations in reproductive synchrony and equality within seed orchards will alter predicted genetic diversity and genetic gain. In this study, highly variable markers were used to assign paternity. They show few pollen contamination and male biased contribution to the seed's progenies. The reason of this unbalanced contribution could be due to the number of clone's copy or to the neighboring. Other studies with increased number of progenies or deeper analysis on flower synchrony and parental assignment must be conducted to get a more precise answer.

PART II

1.BIBLIOGRAPHIC STUDY

Since the seed quality and genetic variability determine the properties of orchard progeny, estimating the optimal seed harvest time is of great relevance. Highly important for seed harvesting is the climate and environment in which the trees are growing, for example elevation, latitude and the growth aspect (Portlock, 1996). Global warming is evidently and rapidly causing biological responses. It is just a matter of time when these changes are going to be noticeable (Aitken, 2008). These global climatic changes can negatively affect seed maturation process which can be devastating for orchard tree breeding. Adaptation to climatic alterations can be observed on molecular level. It is, therefore, intuitive to assume that better understanding of, for example, seed protein patterns can lead to better resolution of eventual environmental changes affecting seed maturation processes.

Conifer seeds consist of three main genetically different structural tissues: 1) seed coat (derived from maternal sporophytic integument); 2) megagametophyte (developed from haploid megaspore), and 3) diploid zygotic embryo (results from the fusion of a male gamete and an egg cell; Chatthai and Misra, 1998). Although progeny trees are under control of both paternal and maternal genotypes, development of embryos and young seedlings is predominantly affected by maternally derived megagametophytes that act as a nutritive tissue (Misra, 1994).

Two major energy and nutritive sources important for the seed germination and plant development are proteins and lipids. Seed storage proteins represent the major source of nitrogen for the germinating seed and developing plantlet so they tend to be rich in asparagine, glutamine,

and arginine or proline. The storage tissue of nitrogen-rich proteins is megagametophyte which begins to accumulate storage proteins after fertilization.

The most of the storage proteins can be divided in insoluble crystalloids and soluble matrix proteins and both are localized in megagametophyte and embryonic axis of maturing seed (Forward, 2000). Seed storage proteins can be classified as albumins, globulins, glutelins or prolamins, respectively (Higgins, 1984). Vicilin- and legumin-like proteins, two globulins, are considered to be the main storage proteins in conifers (Klimaszewska, 2004) and soluble proteins 2S albumine, vicilin- (also called 7S-vicilin-like) and legumin-like proteins (or 11S-legumin-like) comprise 10-30% of storage proteins. In most plants, these proteins are hexamers (legumin-like proteins) or trimers (vicilin-like proteins) with subunit around 45-60 kDa, consisting of 2 peptides (between 27 and 38 kDa) linked by disulfide bridge. Albumin is a monomer of about 25-29 kDa peptide. Abscisic acid plays the crucial role in synthesis of storage proteins in early seed development (Silveira, 2008). In Douglas, the 2S albumin has been identified by its transcript which appears earlier in megagametophyte than in zygotic embryos (Chatthai, 1998). The legumin-like protein has been also identified in the seeds (Lait, 2001). Its maximal synthesis occurs between mid and late cotyledonary stages in *Picea glauca* (Misra, 1991). A study made in Douglas showed that the embryonic part of the seed contains less storage proteins and more housekeeping proteins and maturation and developmental regulators. Proteins in zygotic embryo start to accumulate evidently in the end of the morphogenetic phase, and at this point of seed development it contains energetic reserves originated from megagametophyte (Owens, 1993). As the embryo subsequently maturates, the quantity and complexity of megagametophyte-derived protein bodies increases (Owens, 1993). Since the germination starts in Douglas, the protein content changes both in megagametophyte and embryo axis as a consequence of proteinase activity (Forward, 2000).

Differential protein abundance analysis has established itself as a helpful tool for several purposes. For example, the differences between protein content of somatic and zygotic embryos in culture conditions have brought up several predictive protein markers for somatic embryo development and for the adaptive response of a culture to maturation conditions (Morel et *al.*, 2014). Furthermore, the study of pathogenesis-related (PR) proteins in Douglas-fir-*P. Sulphurascens* pathosystem, has shown that the expression profile of Douglas-fir seed proteins can change as an adaptation to stressful environment (Islam, 2008).

With existing evidence of the environmental impact on the plant development, it can be assumed that climatic conditions and changes can affect the kinetics of protein accumulation of conifer seeds. Since protein content affects seed quality, environmental changes could project on the physiological processes of the plant, particularly seed maturation. The purpose of the protein studies in this research was to determine whether harvest time of the Douglas-fir seeds can be changed depending on the protein content of the seeds. The impact of the temperature treatment on the seed quality was investigated, too. This treatment is applied in commercial seed harvest process. It helps to release the seeds from the cone, and acts positively in the long-term conservation of the seeds. The results could highlight the objectives of future protein analysis and detection of potential protein markers for climatic and environmental changes.

2. RESULTS

2.1. Optimization of the seed number

In order to determine the optimal number of seeds for protein extraction, we did a pretesting with 3 groups of samples differing in the number of seeds. Each group had 5 repetitions and average quantity of µg of proteins per mg of fresh mass was determined using Bradford assay. Each extraction was done using the same volumes of two extraction buffers for each sample. The results are shown in Table 1.

Table 1. The results of extraction efficiency comparison between samples of 2, 3 and 4 seeds. The result is shown as the average quantity of isolated proteins per 1 seed.

NUMBER OF SEEDS PER SAMPLE	QUANTITY (µg of proteins/seed)
2	174.2
3	105.0
4	88.7

Preliminary testing showed that far better extraction efficiency is observed when the extraction is performed with the samples of 2 seeds using the same volume of extraction buffer.

2.2. Protein quantity estimation in seeds and two seed tissues

The protein quantity was estimated for 6 harvest times (Table 2) of temperature-treated and non-treated zygotic embryos, megagametophytes and seeds.

Table 2. The dates of seed collection during 2014, and their designation in the graphs.

THE DATE OF SEED COLLECTION	DESIGNATION IN THE GRAPH
21st July	201
10th August	222
1st September	244
30th September (kept from the 1st Oct at room temperature)	273RT
30th September	273
10th January	375

Figures 1 a and b show the estimation of protein concentrations for zygotic embryos of both genotypes. Although differences in protein concentration for two consecutive harvest times do exist, they are not statistically significant and there is no observed general tendency in difference of protein content.

The protein concentrations of megagametophytes in non-treated and treated samples of both genotypes do not show any general increasing or decreasing tendency (Figures 1 c and d). The differences between non-treated and treated samples in the same harvest time are not statistically significant in neither of genotypes.

Estimated seed protein concentrations are shown in Figures 1 e and f. After initial decrease, a constant increase in protein concentration can be observed for non-treated seeds of genotype x.

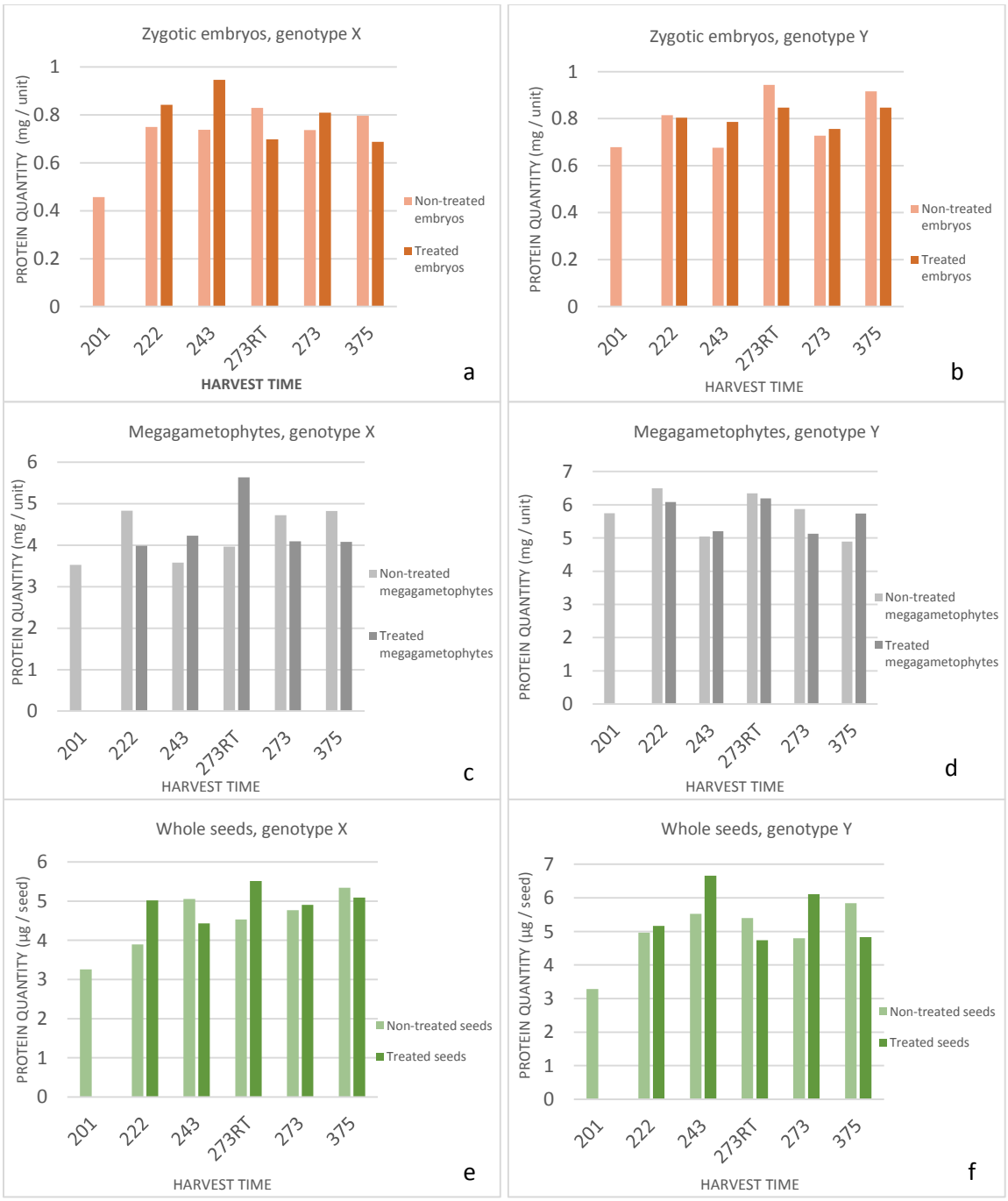


Figure 1. The figure represents the change of protein quantity per unit of zygotic embryo (a and b), megametophyte (c and d) and per seed (e and f) in each harvest time and for both gentoypes.

Altogether, the given results imply several generalities for all three analysed sample types:

- The temperature treatment of the seeds has no real effect on protein content.
- No significant change in protein quantity during maturation process except between the first two dates.

Graphs in Figure 2 represent the mean values for both genotypes and for both treated and non-treated samples. Figures 2 a and b highlight the difference in protein content of the first two harvest times of zygotic embryo and seeds.

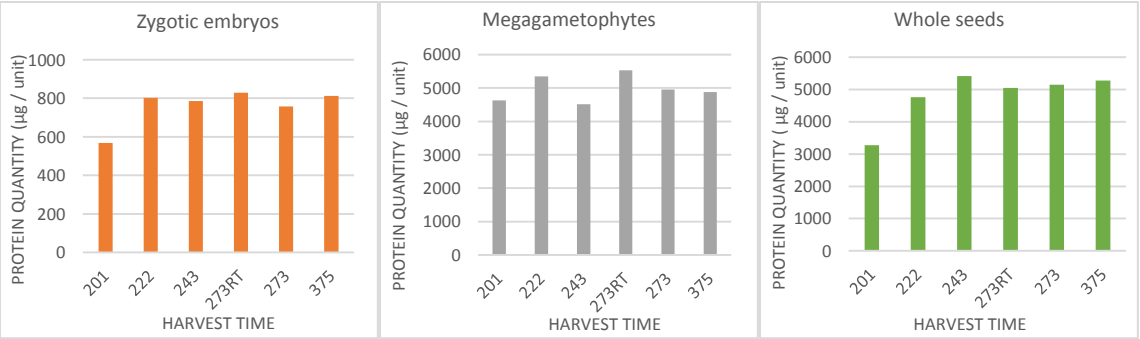


Figure 2. The comparison of the average value of all conditions (average value for both genotypes and both temperature conditions) for all three sample types. Graphs highlight the difference in protein quantity between the harvest days 201 and 222 in zygotic embryos and seeds.

Statistical analysis of the protein content comparison between treated and non-treated samples of the same harvest time of one genotype did not give any significant results. Because of that, we did the statistical analysis with the samples regrouped in several different ways. Firstly, we analyzed only the differences between two consecutive harvest times of the same genotype, with neglecting the temperature treatment difference between samples. Significant differences were observed between the day 201 and 222 in the zygotic embryo of genotype x. In seeds, the differences were significant between the day 222 and 243 harvest time in genotype x and days 201 and 222 in genotype y. In the second regrouping, we analyzed the difference between the same harvest times of two different genotypes. Significant differences were observed for megagametophyte ($P<2.03*10^{-6}$) and seeds ($P<0.034$), but not for zygotic embryo. Finally, we compared protein accumulation according to the harvest times without distinction of the genotypes to obtain more general behavior. Significant differences were then observed in zygotic embryos ($P<3.5*10^{-4}$) and in seeds ($P<2.235*10^{-3}$).

2.3. Protein profiles of the seeds and its tissues

After isolation from the seeds and seed tissues, proteins were separated in denaturing conditions on SDS-PAGE gels. In both seed tissues, there are several hypothetical seed storage proteins which constitute the majority of the total protein content.

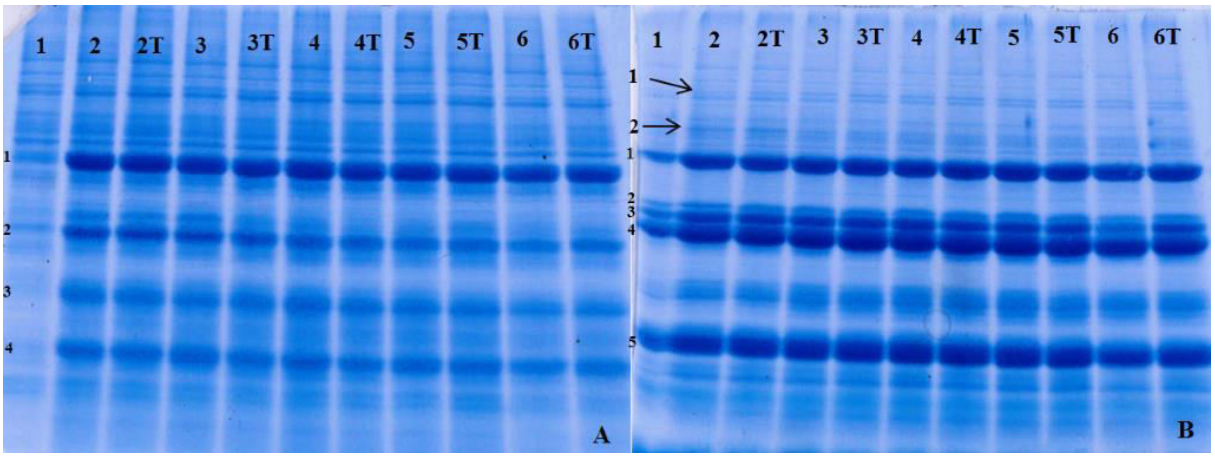


Figure 3. Protein profiles of isolated seed proteins. A shows the protein profile of zygotic embryo for genotype y. Numbers 1-6, +/- T represent harvest times with or without temperature treatment. From A it is visible that the quantity of some proteins during the last phases of seed collection. Numbers 1-4 (left side of the gel) represent major protein bands. B represents protein profile of proteins isolated from megagametophyte of genotype x. Megametophyte proteins appear to be more abundant and their concentration is constant during all of the harvest times. The major proteins are represented with the bands numbered 1-5 (left side of the gel).

In zygotic embryos of both genotypes, one protein appears to comprise the majority of hypothetical storage proteins (Fig. 3A, lane 1), with three less abundant storage proteins (Fig. 3A, lanes 2, 3 and 4). The quantity of all four major proteins is decreasing from the first towards the last harvest time.

There are five major storage proteins present in megagametophyte tissue in each of the genotypes (Fig. 3B, lanes 1-5, genotype y not shown). Proteins in bands 1, 4 and 5 represent the three most abundant proteins. There is no change in protein content of megagametophyte between different harvest times. One of the proteins appears just in the second and remains until last harvest time (Fig 3B, arrow 1) and one of the proteins is present only during the second harvest time (Fig 3B, arrow 2). The overall protein content of megagametophyte is higher than the protein content of zygotic embryo.

According to the literature and the apparent molecular weight of the proteins in the major protein bands, we assume that they are members of the vicilin- and legumin-like group of proteins. To confirm this assumption, we excised these protein bands for the analysis by mass spectrometry.

2.4. Antibody detection of the legumin-like seed proteins

Because the primary antibody (Legumin (1.BB.78)) used for the detection of seed proteins was too diluted, we did not obtain positive results for legumin-like proteins of the seeds. The analysis should be repeated with higher concentration of primary antibody.

2.5. Mass spectrometry analysis of several hypothesized seed storage proteins

The whole set of numbered bands of the gels (Fig 3 A and B) have been submitted to mass spectrometry identification.

3. DISCUSSION AND CONCLUSION

Since there are no significant changes in protein quality and quantity between non-treated and treated seeds for both genotypes and all three analyzed sample types, it can be proposed that:

- The seed germination will not be affected if the seeds undergo temperature treatment (at least up to 40°C overnight). This additional treatment for facilitating the commercial seed harvest process can still be kept in use as it does not have an impact on the seeds;
- The seed quality, regarding its protein composition, does not statistically change between the evaluated harvest times. Nevertheless, the first date seems to be too early for harvest and the seeds seem to still be immature.

However, in order to confirm these assumptions, it is necessary to conduct germination tests with temperature-treated and non-treated seeds collected in different harvest times. Statistically more significant results should be obtained by analyzing more unit repetitions of each tissue, especially between the first date and the others.

The overall protein content of both genotypes appears to be higher in megagametophyte than in zygotic embryo what is understandable since the megagametophyte is the major protein storage tissue.

However, in zygotic embryos, some qualitative and quantitative changes in protein content of particular hypothesized storage proteins can be observed from the electrophoretic results for both genotypes. The decrease of protein content towards the last harvest times might be the consequence of the higher susceptibility of zygotic embryo proteins to degradation by the beginning of the germination process.

The complete protein profile of megagametophyte tissue seems to appear in the second harvest time, with the appearance of high-weight protein (Fig. 3B, arrow 1). This result suggests that seeds can be collected even before commercial harvest time. But, before revealing this information to the seed industry, these results need to be further confirmed and it has to assured that they can be applied each year. It is, therefore, necessary to analyse the samples from another collection drive with contrasted temperatures.

Conclusion

To sum up, we can say that temperature treatment does not really affect the protein content of seeds, so this treatment which facilitates the seed harvest can be left in use. The whole protein set seems to appear three weeks before the current commercial seed harvest time, so this date can be potentially changed after some further investigations and confirmations.

ANNEXES

4. MATERIALS AND METHODS

PART I

4.1. Seed orchard composition

The sample material was collected in an open-pollinated seed orchard *France2,* located in the south-western France in Lavencantière (Lot department). This seed orchard arose from the selection of the best, 10-years old, French massif central's individuals in a progeny test which took place in Argein (near Saint Girons). The selection criteria included: the vigor (total height), the lateness of budburst and form (stem straightness, angle of open branch insertion, and absence of growth in August). The orchard is composed of 134 clones (534 tree individuals, 1-13 individuals per clone). This seed orchard was grafted in 1989 and 1990.

In 2011, all ramets of harvest area were partially girdled to stimulate heavy flowering the following year and to approximately equalize pollen production for all the ramets. That is important for obtaining a panmictic reproduction.

4.2. Phenology and flowering measurements

The flushing phenology and pollen abundance were measured for each ramet in the seed orchard. The flushing phenology state is a good approximation of flowering phenology. Early flushing trees are also early flowering trees. For phenology measurements, general BBCH scale was used (Hack et al., 1992). Notations were made in three times during 2015 spring (30th April, 6th May and 13th May). Each ramet of a certain clone was assigned a value 0-5 according to its bud abundance and state (Table 1).

Pollen abundance was measured in spring 2012 for all the ramets. The scale is 0% to 100%, 0 indicate no male flower on the ramet and 100% more than 3000 male flowers on the ramets. Phenological observations were made one time when all the flowers were whither.

Table 1. The main criteria for assigning a certain bud flushing value to each tree.

VALUE	BRIEF DESCRIPTION
0	dormant buds
1	swollen unflushed buds
2	1-25% flushed buds
3	26-50% flushed buds
4	51-75% flushed buds
5	76-100% flushed buds

4.3. Sample material

The genotypes of all the clones present in the seed orchard were determined prior to offspring genetic analysis. Those results represented adult (maternal and paternal) genotypes.

Based on the labels attached to each tree, we detected 4 mistakes: mislabeled ramets whose genotypes matched a different clone or graft rejection (Jacques, 2014). The data were corrected for this study.

In 2014's spring, each progeny was sown separately in a greenhouse. We collected Douglas-fir needles from 50 individuals of 46 progenies. The plant material of each progeny was stored as separate sample at -80°C. In total, 2150 individuals from 43 progenies were analyzed.

4.4. DNA extraction and genetic analysis

DNA was extracted using NucleoSpin® 96 Plant II kit. The wet weight of sample material was approximately 100 mg per each sample. After DNA extraction, the quantity of DNA was spectrophotometrically measured using NanoDrop. The aliquots were conserved at -20°C until

further use. The genotypes of the 2150 individuals were determined using 11 microsatellite markers (Table 2) divided in 3 multiplex mixes (developed for CGAF laboratory for B.Le Guerroué). DNA was amplified according to the protocol (Annex 2) designed for Guichoux et *al.* (2011) using Mastercycler® thermo-cycler (Eppendorf). The quality and approximate quantity of amplified DNA fragments was determined by gel electrophoresis and ethidium-bromide staining. The DNA extractions and amplifications were done on the 96-well plates in order to facilitate reproducibility of the experiment and sequencing analysis. For the sequencing, 2 µL of PCR product were diluted with 7.7 µL of formamide (Life Technologies) and 0.3 µL GeneScan™ 600 LIZ® Size Standard (Life Technologies). The sequencing was done on a capillary of the automatic genetic analyzer ABI3500.

4.5. Data analysis

4.5.1. The product visualisation and genotyping

The microsatellites were determined by GeneMapper v4.1 and the microsatllite sizes were adjusted using macro Excel AUTOBIN (https://www6.bordeaux-aquitaine.inra.fr/biogeco_eng/Scientific-Production/Computer-software/Autobin).

The genetic analysis was done using Excel macro GeneAlEx 6.501. The analysis enabled the determination of the number of alleles (N_a), the number of effective alleles (N_e), observed heterozygosity (H_o) and expected heterozygosity (H_e), F_{is} and F_{ST} value, as well as the disparity of Hardy-Weinberg equation.

4.5.2. Parentage analysis

The paternity analysis was performed for 43 progenies and their parents using CERVUS 3.03. The utilized parent assignation methods were proposed by Marshall et *al.* (1998) and are based on LOD score (log-likelihood score). LOD score is calculated by the following equation: LOD= T ($g_0/g_m,g_a$) / T (g_0/g_m). T ($g_0/g_m,g_a$) is the transition probability or Mendelian segregation, where g_0, g_m and g_a are gentoypes of the offspring, known mother and alleged father, respectively. T ($g_0/g_m,g_a$) is Mendelian segregation or transition probability, probability of the offspring's genotype given the genotypes of known mother (g_m) and alleged father (g_a), rather than arbitrary male. T (g_0/g_m) is the probability of offspring's genotype given the mother's genotype.

The most probable father was determined utilizing CERVUS 3.0.3. For each analyzed seedling, two most probable fathers were assigned the value Δ as criterion. Δ is the difference between LOD values of two most probable fathers. The Δ estimation was done in CERVUS 3.0.3 performing 10,000 simulations with defined parameters: standard error of 0.01,standard error of 0.01, all the seed orchard's clones (i.e 134) as possible fathers and with the threshold value for the assignment certainty set at "strict" value of 95% and "loose" value of 80%. For the paternity assignment to potential individual, the Δ should have been above critical value. CERVUS analysis was used to estimate the genotyping error rate through detection of null alleles in parentage inference (Kalinowki et al. 2007).

4.5.3. Quantification of genotyping error

Genotyping error can arise from several sources: null alleles due to mutation in priming sites or poor DNA template quality, sutter bands caused by polymerase slippage, human mistake as allele miscalling and mutations generating new alleles. Genotyping error caused primarily by inconsistently amplifying (null and stutter) alleles has been encountered in many studies of pollen flow (Dow and Ashley 1996; Piotti, 2013) leading to observed mismatches between seeds and maternal parent trees. Null alleles, allelic dropout, and stutter bands have all been observed in other studies using microsatellite loci from this marker set (Krutovsky, 2009; Fussi et al. 2013). However, these microsatellites remain the most polymorphic marker set with the highest polymorphic information content available for Douglas-fir, and these markers can still be effective tools for population and progenies genetic analysis. The presence of null alleles was also suggested in allele frequency analysis. However, CERVUS quantifies null allele by identifying excess observed homozygosity which also arises from nonrandom mating or relatedness within a sampled population.

PART II

4.1. Biological material

The plant material, Douglas-fir seeds, was collected in 5 different time periods (21st July 2014, 10th August 2014, 1st September 2014, 30th September 2014, 10th January 2015). After harvest, only those seeds considered full were collected for further analysis. Seeds were collected

from two genetically different Douglas-fir genotypes, x and y genotypes. All seeds, except those harvested in the first period, were divided in two groups, with and without temperature treatment (at least 24 hours at 40°C). The sample material constituted of three different sample types: zygotic embryos, megagametophytes and whole seeds. Embryos and megametophytes were isolated and separated from the seeds and each harvest time was grouped by five units to constitute a sample. Seed samples were constituted of two seeds per each harvest time, with average fresh mass weight about 26 mg. All kinds of samples were stored at -80°C until further use.

4.2. The extraction of total proteins

4.2.1. Sample preparation

The plant material was grounded in the presence of liquid nitrogen at the frequency of 30 for 30 seconds with a tungsten bead (ø5mm). After grounding and before extraction, the samples were stored at -80°C.

4.2.2. Required solutions

Table 2. Composition of the two extraction buffers.

	EXTRACTION BUFFER 1	**EXTRACTION BUFFER 2**
Tris pH 6.8 1M (µL)	450	450
Glycerol (µL)	900	900
SDS 20% (µL)	900	900
PVPP (mg)	180	0
BME (µL)	450	450
H_2O up to 9 Ml	6.3	6.3

4.2.3. The procedure of extraction

After homogenisation, the samples were solubilised in 500 µL of ice-cold first extraction buffer.

After 5' at 95°C, the samples were centrifugated for 10 min at 13500 rpm. Supernatants were collected in separate tubes and the remaining pellet was resolubilized in 450 µL of ice-cold extraction buffer 2 and centrifugated for 10 minutes at 13500 rpm.

Two supernatants were pooled and weighed for the volume estimation. The final centrifugation was performed for clearing the supernatants. During the extraction procedure, samples and supernatants were kept in coolers at 4°C until Bradford assay realised the same day.

4.3. Bradford analysis

4.3.1. The analysis procedure

The protein concentration in each sample was determined using a modified Bradford assay described by Ramagli and Rodriguez (1985) with Coomassie Reagent Dye 5x (Bio-Rad) and bovine serum albumin as a standard.

4.3.2 1D sodium dodecyl sulphate polyacrylamide (SDS-PAGE) electrophoresis

Proteins from total protein extracts were separated on SDS polyacrylamide gel and stained with a colloidal staining.

4.3.3. Gel preparation

The gels were prepared according to the protocol in Table 3. Gel dimensions are 8.6x7.6 x 0.75 mm

Table 3. Composition of the gels realised for protein separation

SOLUTIONS	RUNNING GEL 12%	STACKING GEL 5%
H_2O (mL)	1.65	0.760
Acrylamide/bisacrylamide 30% (mL)	2	0.200
Tris-HCl 1.5 M pH 8.8 (mL)	1.25	0
Tris-HCl 1.5 M pH 6.8 (mL)	0	0.312
SDS 10% (µL)	50	12.5
APS 10% (µL)	50	12.5
TEMED (µL)	2	1.25
Total (mL)	5	1.2

4.3.4. Gel staining

Gels were stained according to the LBLGC protocol (The Laboratory of Woody Plants and Crops Biology, University of Orléans). The scheme of the protocol is shown in Table 4.

Table 4. The procedure steps and composition of each solution for gel staining according to the LBLGC protocol

FIXATION (30´)	ethanol (50%), orthophosphoric acid (2%)
RINSING (3x15´)	H_2O miliQ
SENSIBILIZATION (30´)	methanol (34%), ammonium sulphate (17%), orthophosphoric acid (2%)

COLORATION (2 DAYS, min.)	methanol (34%), ammonium sulphate (17%), CBB G250 (0,033%), orthophosphoric acid (2%)

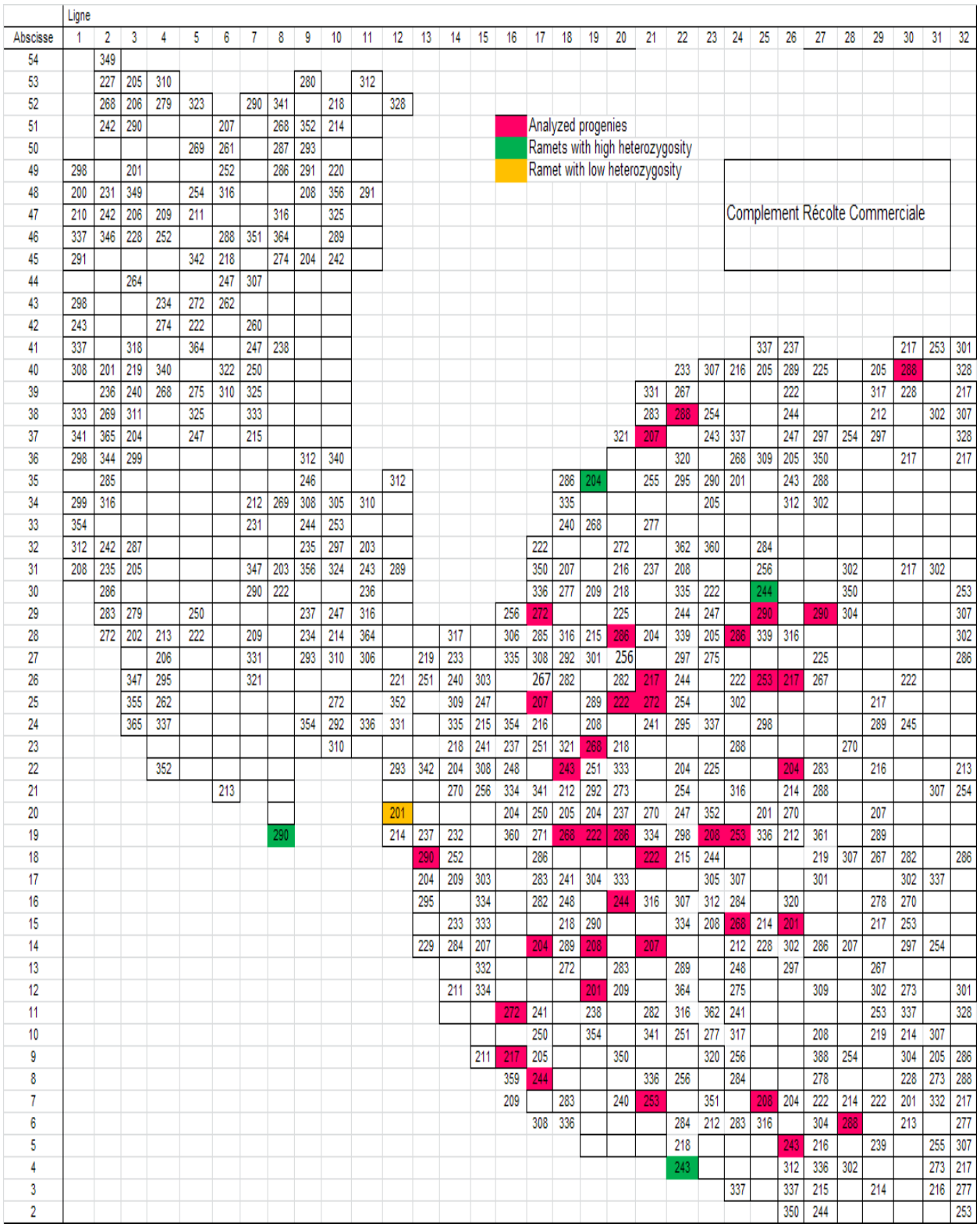
4.4. Western blot analysis

For the Western blot analysis, 40 µg of previously extracted non-treated seed proteins of all six harvest times and the treated seeds of the second, third and sixth harvest time, along with pre-stained mass marker, was denatured in boiling denaturing loading buffer (5' at 95°C). After protein separation by SDS-PAGE gel electrophoresis on a precast 12% gel (Bio-Rad Laboratories, USA), the proteins were transferred to the nitrocellulose membrane using iBlot™ Gel Transfer System (Invitrogen). The transfer was conducted for 7 minutes with applied voltage of 23V. After protein transfer, the membrane was incubated in blocking buffer (3% BSA) for 1 hour. After three washing steps (two times for 5' in TBS-Tween® 20 and one time for 5' in TBS; 100mL of each solution)) the membrane was incubated in the primary antibody (Legumin (1.BB.78): sc-57899, 1:200) for 1 hour on room temperature. After primary antibody incubation, the membrane was washed three times with same solutions as in the previous washing step. The incubation in secondary antibody, anti-mouse alkaline phosphatase, lasted during the night. Before washing in the revelation buffer (TBS+NaCl, 5', 100 mL), the membrane was washed in TBS (3x5', 100 mL). The potential reactions were detected with 10 mL of NBT/BCIP.

4.5. Statistical analysis

The estimated protein concentrations obtained by the Bradford analysis were analysed by the predefined scripts using "R" software. In each analysis, two consecutive harvest times were compared by ANOVA.

Annex 1. The seed orchard map.



Annex 2. The PCR protocol.

PCR mix (µL) :

	Dmix1				**Dmix2**			**Dmix3**			
Markers	**2C3**	**2G12**	**3B2**	**3D5**	**3F1**	**3G9**	**4A7**	**1F9**	**2C2**	**4G2**	**2D4**
Tampon QIAGEN	5	5	5	5	5	5	5	5	5	5	5
Amorce R (10µM)	0.1	0.1	0.1	0.1	0.1	0.15	0.5	0.09	0.08	0.55	0.25
Amorce F (10µM)	0.1	0.1	0.1	0.1	0.1	0.15	0.5	0.09	0.08	0.55	0.25
H_2O (qsp 7.4µL)	2.2	2.2	2.2	2.2	2.2	2.1	1.4	2.22	2.24	1.3	1.9
DNA	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6

In the end, each well contained 2,6 µL DNA and 7,5 µL mix.

PCR programme (Eppendorf machine):

Initiation: 95°C, 15 minutes

Denaturation: 94°C, 30 seconds

Annealing: 56°C, 1 minute

Extension: 60°C, 10 minutes

End: 10°C, ∞

5. ABSTRACT

In order to produce genetically superior seed good enough for reforestation, it is important to get an insight into protein quality and quantity of the seeds and gene flow among trees in the seed orchard. In this study, we tried to determine whether protein content of the Douglas-fir seeds changes as the consequence of the environmental changes (global warming) and to see whether potential changes can be somehow commercially applied. We also investigated the gene flow and genetic structure of the Douglas-fir seed orchard and tried to estimate the cumulative effects of phenology, clonal abundance and spatial distribution of ramets within orchard on genetic diversity of progeny.

ABSTRAIT

Pour produire des graines génétiquement supérieures et de qualité suffisante pour la reforestation, il est important de connaître la qualité des protéines ainsi que la quantité des graines et des flux de gènes entre les arbres du verger à graines. Dans ce travail, nous avons cherché à déterminer si le contenu en protéines dans les graines du Sapin de Douglas (*Pseudotsuga menziesii*) est modifié par les changements environnementaux (réchauffement climatique) et si ces changements potentiels peuvent avoir des applications commerciales. Nous avons également étudié les flux de gènes et la structure génétique dans un verger à graines de Sapins de Douglas et estimé les effets cumulés de la phénologie, de l'abondance des clones et de la distribution spatiale des ramets dans le verger sur la diversité génétique des descendants.

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