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Lack of mother tree alleles in zymograms of *Cupressus dupreziana* A. Camus embryos

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Abstract – Unexpected ploidy levels observed in a previous flow cytometric analysis of *Cupressus dupreziana* endosperm, led us to hypothesize an asexual seed production in this endangered species. We tested here this possible apomixis, studying isozyme variability of endosperms, embryos and seed coats extracted from open pollinated seeds. Electrophoretic bands observed in zymograms of PGI2 and LAP, the only two polymorphic systems, were in agreement with bands observed in *C. sempervirens* zymograms. Zymograms of *C. dupreziana* endosperms were identical to those of the diploid maternal tissue, as observed in *C. sempervirens*. Hypothesizing a codominant nuclear DNA control for both PGI2 and LAP, a similar electrophoretic expression of these systems in endosperm and embryo, and the absence of modifier genes, the lack of endosperm bands in most embryo zymograms was interpreted as the result of a strictly paternal origin of embryo nuclear DNA in *C. dupreziana* seeds.

Cupressus dupreziana / isozyme / reproduction / gymnosperm / paternal inheritance

Résumé – Absence d'allèles maternels dans les zymogrammes des embryons de *Cupressus dupreziana* A. Camus. De précédents travaux portant sur les niveaux de ploïdie de l'endosperme des graines de *Cupressus dupreziana*, nous avons conduit à émettre l'hypothèse d'une reproduction apomictique chez cette espèce menacée. Cette hypothèse a été ici testée par l'étude de la variabilité enzymatique d'endospermes, d'embryons et de téguments de graines issues de pollinisation libre. Les zymogrammes observés pour les deux systèmes polymorphes, PGI2 et LAP, sont conformes à ceux observés chez *C. sempervirens*. Les zymogrammes des endospermes de *C. dupreziana* sont identiques à celui de l'arbre mère, comme précédemment observé chez *C. sempervirens*. La plupart des zymogrammes produits par les embryons des graines de *C. dupreziana* ne contiennent pas les bandes électrophorétiques des endospermes correspondants. Chez tous les gymnospermes étudiés, PGI2 et LAP sont des marqueurs codominants, sous contrôle génétique nucléaire. Chez *C. sempervirens* l'expression de ces systèmes est indépendante des tissus (endosperme ou embryon) et aucun gène modificateur n'est connu. Dans ce contexte, nous formulons l'hypothèse d'une origine strictement paternelle de l'ADN nucléaire des embryons.

Cupressus dupreziana / isozyme / reproduction / gymnosperme / hérédité paternelle

1. INTRODUCTION

In gymnosperm seeds, the embryo is generally surrounded by a haploid tissue called endosperm (or megagametophyte). However we recently demonstrated that in some Cupressaceous species, and especially in Mediterranean cypresses, the endosperm exhibits multi-

ple levels of ploidy [33]. *Cupressus sempervirens* L. originating from the eastern Mediterranean basin, and *C. atlantica* Gaussen, an endemic species from the Atlas mountain in Morocco, exhibited similar profiles of endosperm DNA contents, corresponding to even and odd levels of ploidy (1C, 2C, 3C, 4C, 5C, 6C...). We demonstrated that these multiple ploidy levels were

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due to nuclei fusion during megagametogenesis [13]. In gymnosperms, the endosperm derives from the megagametophyte that develops, after meiosis, from generally one but sometimes several (four in *C. sempervirens*) megaspore(s) [14]. Through archegonia, the megagametophyte produces female gamete(s).

Multiple ploidy levels were also observed in the endosperm of *Cupressus dupreziana* A. Camus, the other Mediterranean cypress but, unexpectedly, only even levels [35]. *C. dupreziana* is an endangered tree species from the Tassili N'Ajjer desert, Algeria [3, 36]. Approximately 150 trees are still surviving in this natural area [10]. The absence of 1C DNA nuclei in *C. dupreziana* endosperm raises the question of existence (and origin) of female gametes. In order to test an hypothetical asexual seed production (apomixis), isozyme variability was studied in 5 open-pollinated families.

2. MATERIALS AND METHODS

Seeds were extracted from mature cones collected from five 17 year old *C. dupreziana* trees (referred as D38, D28, OO1, J1 and DD5) planted in a *ex-situ* collection in the Esterel Mountain (Southern France) distant by 1.5 km from the nearest cypress stand which reduces the possibility of exogenous pollination. This plantation is composed of 275 *C. dupreziana* trees representing 35 clones (5 ramets per clone) and 6 open pollinated progenies. Genetic diversity of this material is unknown. Trees OO1, J1 and DD5 are three different clones, while D38 and D28 belong to the same progeny.

Seeds from one *C. sempervirens* tree were used as control. In order to confirm the diploid-like maternal origin of the endosperm [29], we also analyzed endosperms and seed coats of immature seeds collected from another *C. dupreziana* tree referred as RUS.

Isozyme analyses were performed on samples (embryo, endosperm or seed-coats) extracted from single seeds. Isozymes were revealed using horizontal starch gel electrophoresis. Electrophoresis and staining procedures followed those of Papageorgiou [28]. Four enzyme systems were scored: Leucine aminopeptidase (LAP, EC 3.4.11.1), Malate dehydrogenase (MDH, EC 1.1.1.37), Nicotinamide adenine dinucleotide dehydrogenase (NDH, EC 1.6.99.3) and Phosphoglucose isomerase (PGI, EC 5.3.1.9). Zones and bands in each system were coded according to their decreasing mobility, starting from 1 for the fastest zone or band.

3. RESULTS

Most seeds were empty in all *C. dupreziana* seed lots (table I). One seed, from tree DD5, had an endosperm but no embryo.

No variation was observed in MDH and NDH. Banding patterns were similar to those of the *C. sempervirens* control. PGI and LAP were polymorphic and thus usable for analyzing isozyme variability among endosperm, embryo and seed coat samples. Observations were the following:

PGI: two zones of activity were observed. Variation was found only in the slowest zone (PGI2) where two single-banded phenotypes were observed in all but one *C. dupreziana* samples while a three-banded phenotype was observed in all *C. sempervirens* endosperms used as control (figure 1). All *C. dupreziana* endosperms

Table I. Number of studied seeds collected from 6 *C. dupreziana* and 1 *C. sempervirens* trees.

Mother tree code	Number of dissected seeds	Number of filled seeds
<i>C. dupreziana</i>	D38	200
	D28	184
	OO1	173
	J1	244
	DD5	152
	RUS	221
<i>C. sempervirens</i>	12	8 (66%)

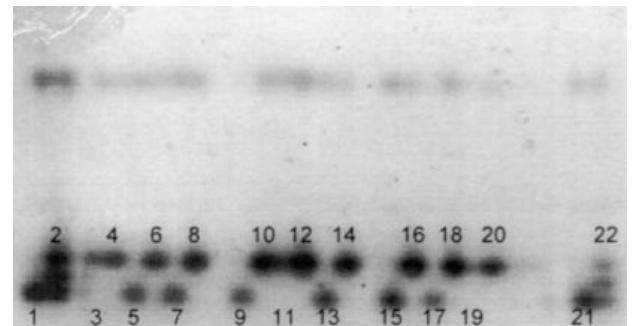


Figure 1. Example of a PGI gel. The monomorphic upper zone corresponds to PGI1 and the lower polymorphic zone to PGI2. Samples 1, 2, 21 and 22: *C. sempervirens*.; all other samples: *C. dupreziana*. Embryos: odd samples; corresponding endosperms: even samples. All *C. dupreziana* samples have a single-banded phenotype for PGI2. In 6 of the 9 *C. dupreziana* seeds, the endosperm allozyme is absent in the embryo zymogram. *C. sempervirens* endosperms show a three-banded phenotype corresponding to a heterozygous genotype.

produced phenotype 1, while embryos produced either phenotype 1 (8 samples out of 32), phenotype 3 (23/32) or phenotype 1-2-3 (1/32). For each seed lot, occurrence of the three observed phenotypes is described in *table II*.

LAP: one zone of activity was observed. Band 1 was only observed in *C. sempervirens* endosperms, while the 3 other bands produced 3 different phenotypes among the *C. dupreziana* samples (*table III*). All *C. dupreziana* endosperms produced phenotype 3-4 which was never observed in embryos. Embryos produced phenotype 2 (22 samples out of 30) or 2-3 (8/30).

4. DISCUSSION

4.1. Empty seeds

The very low percentage of filled seeds observed in the 6 *C. dupreziana* seed lots analyzed is in agreement with previous observations and could be due to abnormal sexual reproduction [35].

4.2. Genetics of isozyme systems

To our knowledge, there is no published results about variability of biochemical markers in *C. dupreziana*, either with isozymes or with other markers. *C. sempervirens* is the closest species for which isozymes studies are available. Both LAP and PGI enzyme systems were analyzed in *C. sempervirens* progenies and/or provenances [18, 21, 28, 30, 31]. Description of zymograms and inheritance of allozymes were tested by Giannini et al. [18] using self-pollinated families and by Papageorgiou et al. [30, 31] using open-pollinated families. These authors agreed with the following genetic interpretation that we considered to be true for *C. dupreziana* as well:

PGI2 is a dimeric enzyme with a monolocus biparental codominant inheritance. Two alleles produced three phenotypes: 2 single-banded phenotypes for homozygotes and one three-banded phenotype for heterozygotes. In our study, all bands were observed.

LAP is a monomeric enzyme with a monolocus biparental codominant inheritance. Six bands corresponding to six alleles were revealed. Thus homozygotes exhibited only one band while heterozygotes exhibited two bands. In our study, only four bands were observed and interpreted as 4 alleles.

Table II. Number of samples belonging to one of the 3 PGI2 phenotypes observed. Phenotype numbering refers to decreasing band mobility.








Sample	Mother tree	Tissue	Number of samples per phenotype			
			1	3	1-2-3	unreadable
						
D38		embryo	0	6	0	0
		endosperm	6	0	0	0
D28		embryo	2	5	1	0
		endosperm	8	0	0	0
OO1		embryo	1	2	0	0
		endosperm	3	0	0	0
J1		embryo	3	6	0	0
		endosperm	9	0	0	0
DD5		embryo	2	4	0	0
		endosperm	6	0	0	1
<i>C. sempervirens</i>		embryo	0	3	2	0
		endosperm	0	0	5	0

Table III. Number of samples belonging to one of the 4 LAP phenotypes observed. Phenotype numbering refers to decreasing band mobility.

Sample	Mother tree	Tissue	Number of samples per phenotype				unreadable
			12	2	23	34	
							
D38		embryo	0	4	2	0	0
		endosperm	0	0	0	6	0
D28		embryo	0	4	2	0	2
		endosperm	0	0	0	8	0
OO1		embryo	0	1	2	0	0
		endosperm	0	0	0	3	0
J1		embryo	0	7	2	0	0
		endosperm	0	0	0	9	0
DD5		embryo	0	6	0	0	0
		endosperm	0	0	0	6	1
RUS		seed coats	0	0	0	4	0
		endosperm	0	0	0	8	0
<i>C. sempervirens</i>		embryo	4	4	0	0	0
		endosperm	8	0	0	0	0

4.3. Seed tree genotypes in endosperms

Due to the tetrasporic origin of the megagametophyte [14] and nuclei fusion during its development [13], the endosperm of *C. sempervirens* exhibits an enzymatic phenotype identical to the seed tree phenotype. Thus the diploid patterns produced by the *C. sempervirens* endosperms used as control (*tables II and III*) show that this seed tree is heterozygous for both systems LAP and PGI2.

Endosperms and seed coats extracted from seeds of *C. dupreziana* RUS produced an identical two-banded phenotypes for LAP, the only system tested. This result supports the hypothesis of a diploid maternal contribution to the megagametophyte of *C. dupreziana* as shown for *C. sempervirens*. Thus all *C. dupreziana* seed trees have the same genotypes, homozygous for allele 1 in PGI2 and heterozygous for alleles 3 and 4 in LAP. Such lack of genotype diversity could be due to the low sample size.

4.4. Embryo genotypes of *C. dupreziana* seeds

The alleles present in the embryos and absent in the endosperms (seed trees) indicate the existence of other genotypic combinations among pollinating trees. Due to the geographic isolation of the plantation, these alleles should mainly come from the neighbouring *C. dupreziana* trees.

Many embryos analyzed in the present study exhibit zymograms where band(s) of the corresponding endosperms were absent: 23 embryos out of 32 for PGI2 and 22 embryos out of 30 for LAP. These two enzymatic systems were reported to be under nuclear DNA control with codominant alleles not only for *C. sempervirens* but also, to our knowledge, for all gymnosperms studied [1, 7, 8, 12, 15, 19, 22, 25, 32, 37, 38]. All enzymatic systems tested in *C. sempervirens*, (including PGI and LAP) were similarly expressed in endosperm and embryo tissues [29]. The existence of a gene modifying electrophoretic properties, as found for MDH in a few species [6], has never been demonstrated for PGI and LAP. Hypothesising, as in *C. sempervirens*, 1) a nuclear codominant genetic control, 2) a similar enzymatic tissue activity and 3) the absence of modifier genes, the frequent lack of maternal alleles in embryo can be best explain by a strictly paternal origin of embryo nuclear DNA in *C. dupreziana* seeds.

In most *Cupressaceae* studied, such a paternal inheritance was already observed for both chloroplast and mitochondrial DNA, while in Pinaceae mitochondrial DNA is usually maternally inherited [9, 26]. But, to our

knowledge, paternal inheritance of the whole nuclear DNA was never reported.

4.5. Androgenesis and/or paternal apomixis

Paternal inheritance of embryo nuclear DNA implies embryogenesis from nuclei of the male prothallus that is produced by germination of a pollen grain.

Production of haploid embryos from anther culture is a well known phenomenon, called androgenesis [5, 16], and frequently used in plant breeding. *In planta* androgenesis would imply the production of haploid (or dihaploid) embryos. However, a previous study revealed that *C. dupreziana* embryos were diploid [35] and some of the enzymatic phenotypes observed in the present study corresponded to heterozygous embryos. A “paternal sexual reproduction” could also be hypothesized, where nuclei fusion occurs between male gametes produced by one or several pollen grains. However, recent analyses revealed that *C. dupreziana* pollen contains unreduced diploid nuclei [34]. This unknown feature in gymnosperms is in agreement with previous morphological observations on the extraordinary large [17] or aberrant [2] size of *C. dupreziana* pollen. It is the largest observed pollen in the genus *Cupressus* (38 μ vs. 28 μ for *C. sempervirens*). We thus hypothesize that unreduced male gametes are responsible for the development of diploid embryo in *C. dupreziana*.

If the definition of apomixis is not limited to embryo development from maternal tissue, but means asexual formation of seed, as commonly accepted [27], then *C. dupreziana* may be one apomictic species. Apomixis is frequently observed in angiosperms and mostly in polyploid species. At least 300 species in 35 families (mainly Gramineae, Asteraceae, Compositae, Rosaceae and Rutaceae) are concerned [20, 27]. It has never been observed in *in planta* reproduction of gymnosperms [24] although some references report apomictic type deviations from the sexual cycle of conifers [4, 11, 23].

In comparison with “maternal apomixis”, *C. dupreziana* embryo development from paternal tissue (or at least paternal nuclear DNA) and without fertilization, would be a unique case of “paternal apomixis”. The biological significance and adaptive “benefits” of this unexpected feature are not clear. It may be the expression of a trait of survival for a species no more able to produce female gametes, as inferred from the absence of 1C nuclei in endosperm.

Further observations using molecular markers controlled by nuclear and cytoplasmic DNA, associated with cytological observations of female and male tissue development, before and during embryogenesis, should

make it possible to determine the exact contribution of father and mother trees in *C. dupreziana* seed production, and to know if normal fertilisation sometimes occurs in this species.

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